The *Caenorhabditis elegans* Homolog of the Opitz Syndrome Gene, *madd-2/Mid1*, Regulates Anchor Cell Invasion during Vulval Development

and

Anchor Cell Polarity Negatively Regulates Vulval Induction

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1 Preface

In retrospect, what I ended up studying during my PhD is the role of one single cell in *C. elegans* development. A cell, in fact, that ceases to exist before the worm becomes adult. Yet this cell is peculiarly important and has a multitude of signalling and other tasks and became known even among non-*C. elegans* researchers recently because of one of its many roles. It is the anchor cell, or short AC, of *C. elegans*, and that one role is of course that of tissue boundary breaching. It does this during a process called AC invasion and also I worked on it. As such it will be covered in the first part of my thesis. However, there is more to the AC than just invasion and tissue breaching. As mentioned, it is involved in many signalling events, but also serves as the linchpin of vulval and uterine development, both as organiser and executor. But generally, the AC is not seen as an anchor of development, but just as this one cell that has certain characteristics of metastasising cancer cells. This is understandable to a certain degree. After all, a lot of research grants are awarded to studying cancer. But the AC does much more, and the ways in which it coordinates all these different roles, how it switches between them and whether there might seem some underlying mechanism that enables it to fulfill these many roles efficiently has been ignored to a large degree. In my view, *C. elegans* is a useful model to study pathways and even specific processes relevant to human diseases such as cancer, but its true strength as a model is the ability to study these pathways and their involvement and role during development and to construct an in vivo network of how different pathways and genes interact when building an organism starting from one single cell. For all of this, *C. elegans* offers great genetics and more and more tools became available over the last few years that allow for ever more sophisticated experiments. The understanding we have of the various pathways and the manner in which they are working in *C. elegans* is such that it is easy to jump from one question to the next and come up with ever more intricate experiments designed to answer always more detailed questions supposed to highlight the one unique aspect of a given process in this one particular situation. It is in many cases well-founded half-complete knowledge that facilitates these very interesting, stimulating and fun thought experiments. However, it is in my experience often the case that either the question we are asking will not give the answer to the question that we are actually interested in or that there are much more effective, but also much more mundane, ways to pursue the goal. It is the ability to take a step back from the intricate details that so easily captivate and stimulate our fascination and our interest and to look for another approach that might be less fun and interesting but more efficient and promising that was one of the

more valuable experiences pursuing research during the course of this thesis. Another issue that is at the core of the second part of my thesis is the role of dogmas in biology, and possibly science in general. But I do not mean the spelled and deliberately articulated dogmas of, e.g. molecular biology, but dogmas that we absorb in the course of our basic education and that are never revealed and addressed as what they are. I'm referring to the idea that most, if not all secreted proteins are thought to form a gradient once they are released from the cell they were produced in. This assumption, and in most cases it is an assumption, is based on the idea that proteins diffuse with random brownian motions. And whilst this is certainly true in most cases, just because the motion of a protein is random, does not mean that its distribution will be uniform. It is not just the motion that is important to determine distribution but also in which way it is released, where the motion starts. And it is this unmentioned dogma that I try to maybe soften up a bit and at least make it a recognized dogma by studying one case during vulval development where the postulated graded distribution of a growth factor, LIN-3/EGF, is essential in ensuring a wild-type pattern of vulval induction. It is the ability to take a step back also here that is crucial in my experience. Not from the thought labyrinth but from all the ingrained knowledge and established principles in order to examine them for what they are. Because they are just ideas of how we think everything works and not how everything actually works. Famously, there is no way to verify any model, only falsification is possible. And we must keep on trying to falsify any and all models to make sure they are the best possible representation of our knowledge. Even if this is mostly theoretical scientific philosophy, the principle of examining what is knowledge and what is assumption based on knowledge is a crucial, if very difficult part of the scientific method. And so it is only fitting that it is in part this principle that is responsible for the second part of this thesis, when I try to re-examine an established model to add significance and evidence to a central part of vulval development.

Contents

2 Zusammenfassung

Der kleine Fadenwurm *Caenorhabditis elegans* wird als Modellorganismus in vielen verschiedene Forschungsfeldern benutzt. Die Eigenschaften von einzelnen Zellen (Zellbiologie), wie der erwachsene Organismus aus einer befruchteten Eizelle entsteht (Entwicklungsbiologie), wie Nerven kommunizieren und das Verhalten kontrollieren (Neurobiologie), und sogar höhere kognitive Funktionen, wie zum Beispiel Lernen, werden alle in diesem kleine Wurm untersucht. Der Organismus von *C. elegans* ist simpel und die Anzahl Zellen die er hat ist klein und präzise definiert. Jede Zelle kann während ihres gesamten Lebens verfolgt werden und was immer sie tut, kann genau vorhergesagt werden. Dies ist möglich aufgrund der fixen Zellabstammung von *C. elegans*.

Während meiner Studien versuchte ich zu verstehen, wie ein kleiner Teil des hermaphroditischen Wurms, sein Organ zum Eier legen oder Vulva, entsteht. Dieser Prozess wird normalerweise als Vulva-Entwicklung bezeichnet. Um genau zu sein, studierte ich bloss einen kleinen Teil dieses kleinen Teils der Entwicklung von *C. elegans*. Meine Studien waren für den grössten Teil auf bloss eine Zelle fokussiert. Diese Zelle heisst Ankerzelle. Die Ankerzelle hat eine zentrale Rolle während der Entwicklung der Vulva und auch des Uteruses von *C. elegans*. Ihr Name verdankt die Ankerzelle vermutlich der Tatsache, dass sie die Position der Vulva festlegt/verankert und dann das Organ zum Eier legen mit dem zum Eier produzieren verbindet. Dies sind auch die zwei am besten beschriebenen Funktionen der Anker Zelle: Die Induktion der Vulva und Ankerzellinvasion. Weil es in dieser Dissertation vor allem um die Ankerzelle geht, werde ich die Biographie dieser Zelle kurz vorstellen.

Die Ankerzelle ist eine Zelle der somatischen Gonade und hat eine von zwei möglichen Zellabstammungen: Z1.ppp oder Z4.aaa. Welche genau wird zufällig entschieden, und zwar vom Notch Signalweg in einem Prozess, der laterale Inhibition genannt wird. Laterale Inhibition ermöglicht es mehreren Zellen mit gleicher Kompetenz, eine Zelle unter sich zu bestimmen und auszuwählen. Nachdem die Ankerzelle bestimmt wurde, beginnt sie einen Homolog des epidermalen Wachstumsfaktors von Säugetieren, LIN-3/EGF genannt, zu produzieren. LIN-3/EGF wird von der Ankerzelle sekretiert und von einer Gruppe von sechs gleich kompetenten Zellen, den Vulva Vorläufter Zellen, wahrgenommen. Alle diese Zellen können sich als Teil der adulten *C. elegans* Vulva entwickeln, falls sie das LIN-3/EGF Signal spüren, das von der Ankerzelle kommt. In einem normalen wild-typ Tier werden nur drei der sechs Vulva Vorläufer Zellen einen Teil der adulten Vulva bilden. Diese drei Zellen befinden sich nebeneinander und ihre Namen sind P5.p, P6.p, und

P7.p. Die mittlere P6.p Zelle ist normalerweise der Ankerzelle am Nächsten und bekommt daher am meisten LIN-3/EGF. Aufgrund dessen wird P6.p die 1° Vulvazelle. Die anderen zwei Zellen, P5.p und P7.p, bekommen weniger LIN-3/EGF von der Ankerzelle, aber auch ein Notch Signal von P6.p. Deshalb werden diese Zellen 2° Vulvazellen. Nachdem die Ankerzelle die Entwicklung der Vulva durch das Bestimmen der Vulvazellen gestartet hat, verbindet sie den Uterus, wozu auch sie gehört, mit der Vulva. Der Prozess der das Herstellen dieser Verbindung startet wird Ankerzellinvasion genannt.

Dieser Prozess is sehr speziell und selten während der Entwicklung, weil er eine Zelle involviert, die Gewebsgrenzen überschreitet. Im Falle der Ankerzellinvasion überschreitet die Ankerzelle aus dem Uterus die Grenze, welche aus zwei Basal-Laminas besteht, und dringt in das Gewebe der Vulva ein. Dieser Prozess ist aus folgendem Grund sehr interessant für uns: Es ist ein Prozess, welcher der Metastasierung von Krebszellen sehr ähnlich ist. Tatsächlich konnte gezeigt werden, dass die Gene, welche die Ankerzellinvasion regulieren, auch die Fähigkeit von Krebszellen zu Metastasieren beeinflussen können. Aber die Ankerzelle hat sogar noch mehr zu tun. Nach der Invasion wird sie gebraucht, um die Entwicklung der 2° P7.p Zelle richtig zu orientieren. Sie tut dies durch das Senden eines Wnt Signals. Als nächstes instruiert die Ankerzelle das Formen einer Gruppe von spezialisierten Uteruszellen. Diese Zellen werden die Vulva mit dem Uterus verbinden. Das Signal, welches diese Uteruszellen bestimmt, ist wiederum Notch. Die letzte Aufgabe der Ankerzelle während der normalen Entwicklung ist es, den Platz freizugeben, durch welchen die Eier gelegt werden. Zu diesem Zweck fusioniert sie mit anderen Uteruszellen und formt so die uterine Nahtzelle. Dieser Prozess lässt eine sehr dünne Zellschicht zurück, die den Uterus von der Vulva trennt. Diese Zellschicht ist das *C. elegans* Hymen. Es wird brechen, sobald das erste Ei gelegt wird.

Während meiner Studien habe ich mich mit zwei von diesen Aufgaben der Ankerzelle detailliert befasst. Der erste Teil meiner Studien hat sich um die Ankerzellinvasion gedreht. Ich beschrieb das Gene *madd-2*, welches nach einem anderen Prozess, den es reguliert, der Muskelarm-Ausstreckung, benannt ist. Als ich dieses Projekt begann, war bereits bekannt, dass das *C. elegans Netrin* Gen *unc-6* die Ankerzelle während der Invasion lenkt. Wenn *unc-6* fehlt, weiss die Ankerzelle nicht, wohin sie eindringen soll und die Invasion findet nicht statt. Beim Untersuchen von *madd-2* entdeckten wir, dass ein Fehlen von *madd-2* die Ankerzelle ebenfalls vom Eindringen abhält. Als wir dann aber die Ankerzelle zwischen *unc-6* und *madd-2* Mutanten verglichen, sahen wir einen grossen Unterschied. Während der Invasion macht die Ankerzelle Ausstülpungen in Richtung von UNC-6 und der Vulva. Wenn UNC-6 fehlt, dann macht die Ankerzelle im Grossen und Ganzen keine

Ausstülppungen. Überraschenderweise aber sahen wir, dass die Ankerzelle von *madd-2* Mutanten eine erhöhte Anzahl Ausstülpungen hatte, und auch nicht bloss in die Richtung von UNC-6. Als wir die *unc-6* und *madd-2* Mutationen miteinander kombinierten, wurde der Invasionsdefekt nicht verstärkt sondern vergleichbar mit *madd-2* Einzelmutanten. Sogar ohne UNC-6 und ohne Vulva formte die Ankerzelle ohne *madd-2* immer noch Ausstülpungen. Aufgrund dessen vermuten wir, dass *madd-2* die Aufgabe hat dafür zu sorgen, dass UNC-6 und die Vulva die Ankerzelle lenken können und ungelenkte Ausstülpungen unterdrückt werden. Aufgrund der Eigenheiten von metastasierenden Krebszellen, der Ähnlichkeit zwischen Ankerzellinvasion und Metastasierung und dem tödlichen Ausgang von Metastasenbildung, ist der von uns untersuchte Prozess theoretisch extrem wichtig. Praktisch fehlt eine konkrete Verbindung zwischen *madd-2* und einer Förderung oder Hemmung von Metastasierung. Bloss in einer einzigen Studie, welche Zellinvasion und Genexpression korreliert, kann solch eine Verbindung gefunden werden. Gute Daten und Hinweise um diese mögliche Aufgabe von *madd-2* zu untermauern fehlen.

Der zweite Teil meiner Studien befasste sich damit, wie die Ankerzelle während dem Bestimmen der Vulvazellen LIN-3/EGF sekretiert. Die Induktion der Vulva ist ein gut untersuchtes Beispiel dafür, wie Zellen instruiert werden sich zu teilen, zu differenzieren und zu wachsen. Wie bereits erwähnt, sind die zwei Hauptsignale während dieses Prozesses LIN-3/EGF und LIN-12/Notch. Es ist die Ankerzelle, welche LIN-3/EGF produziert. Die 1° P6.p Zelle bekommt am meisten LIN-3/EGF und ihre Nachbarzellen P5.p und P7.p bekommen weniger. Dies ist ein Grund dafür, dass wir die Verteilung von LIN-3/EGF als Gradient vermuten. Von Studien über die Ankerzellinvasion wissen wir, dass die Ankerzelle sehr stark polarisiert ist und die meisten Proteine und Lipide in der Membran nicht gleichmässig in der Zelle verteilt sind, sondern an einem Ort konzentriert sind. Wir begannen uns zu fragen, ob die Polarität der Ankerzelle während der Induktion der Vulva wohl eine Rolle spielt und ob die Polarität die Verteilung von LIN-3/EGF beeinflusst. Von früheren Experiment ist bekannt, dass wenn die Vulva Voräuferzellen empfindlicher für das LIN-3/EGF Signal sind, die Verteilung von LIN-3/EGF bestimmt, welche Zellen induziert werden. Falls LIN-3/EGF zu anderen Zellen als P5.p, P6.p, und P7.p gelangt, können diese anderen Zellen dann auch Vulvazellen werden. So ein Fall, in dem mehr als drei Zellen versuchen, Vulvazellen zu werden, wird Multivulva genannt. Für den Wurm ist das nicht wünschenswert, da die zusätlichen Zellen die normale Vulvaentwicklung stören. Wir entdeckten, dass ein Ändern der Ankerzellpolarität auch die Verteilung von LIN-3/EGF ändert. Wenn die Vorläuferzellen der Vulva dann noch LIN-3/EGF empfindlicher sind, kann das zu einer Multivulva führen.

Wenn die Empfindlichkeit gegenüber LIN-3/EGF der Vorläuferzellen normal ist, kann eine veränderte LIN-3/EGF Verteilung beeinflussen, welche Zellen Vulvazellen werden. Konkret heisst dass, das P4.p, P5.p, und P6.p Vulvazellen werden können anstatt P5.p, P6.p, und P7.p. Das ist etwas, das während der normalen Entwicklung nie passiert, aber ob es auch einen Einfluss darauf hat, wie die Eier gelegt werden, ist nicht bekannt. Das Wichtige an unserer Entdeckung ist, dass die Verteilung von LIN-3/EGF nicht zufällig ist, sondern reguliert werden muss. Wie das genau geschieht, ist noch unklar. Grundsätzlich jedoch wird das Senden von Signalen in den Zellen untersucht, die das Signal erhalten, wie zum Beispiel die Vorläuferzellen der Vulva. Produktion und Senden des Signals wird grössenteils vernachlässigt oder sogar ignoriert. Wir zeigen, dass die sendende Zelle nicht nur ändern kann, wie das Signal gesendet wird, sondern auch, dass sie eine wichtige Rolle dabei spielt, welches Zellschicksal die empfangende Zelle annimmt.

Die Ankerzelle ist eine Zelle mit vielen verschiedenen Aufgaben während der Entwicklung. In meiner Dissertation beschreibe ich zwei dieser Aufgaben im Detail und trage zu ihrem Verständnis bei. Die Ankerzelle wird momentan vor allem als Modell für Zellinvasion benutzt, weil das medizinisch sehr relevant ist. Aber die Ankerzelle macht nicht bloss Invasion und sie ist auch mehr als nur die Zelle, welche das Signal für die Induktion der Vulva produziert. Die Ankerzelle kann als Modell dienen, um nicht nur andere Prozesse während der Entwicklung zu untersuchen, sondern auch wie diese verschiedenen Prozesse von einer einzigen Zelle ausgeführt werden.

3 Summary

The small nematode *Caenorhabditis elegans* is used as a model organisms for many different areas of research. The characteristics of single cells (cell biology), how an adult organism is formed starting from a fertilized egg (developmental biology), how nerves communicate and control behaviour (neurobiology), and even higher cognitive functions, such as learning, are all studied in this little worm. The organism of *C. elegans* is simple and its cell number is small and precisely defined. Every cell can be followed during its lifetime, and whatever it does can be accurately predicted too, because the cell lineage of *C. elegans* is fixed.

During my studies, I tried to understand how a small part of the hermaphrodite worm, its egg-laying organ or vulva, is formed. This process is commonly called vulval development. To be precise, I only studied a small part of this small part of the development of *C. elegans*. For the most part, my studies were focussed on one cell only. This cell is called the anchor cell. During the development of the vulva and also the uterus of *C. elegans*, the anchor cell is central. Its name most likely stems from the fact that it anchors the position of the vulva and then connects the egg-laying to the egg-producing organ. These are also the two best-characterized functions of the anchor cell: Vulval induction and anchor cell invasion. But, because this thesis is mostly about the anchor cell, I will give a short but full biography of this cell.

The anchor cell is a cell of the somatic gonad and has one of two possible lineages: Z1.ppp or Z4.aaa. Which one exactly is decided randomly using the Notch signalling pathway in a process called lateral inhibition. Lateral inhibition enables multiple, equally competent cells to decide and select one cell among themselves. After the anchor cell has been selected, it produces a homologue of the mammalian epidermal growth factor called LIN-3. LIN-3/EGF is secreted from the anchor cell and sensed by a group of six equally competent cells, the vulval precursor cells. These cells can all develop to be a part of the adult *C. elegans* vulva if they sense the LIN-3/EGF signal coming from the anchor cell. In a normal wild-type animal, only three of the six vulval precursor cells become part of the adult vulva. These three cells are adjacent and their names are P5.p, P6.p, and P7.p. The central P6.p cell is usually closest to the anchor cell and thus receives most LIN-3/EGF. Because of that, P6.p becomes the 1° vulval cell. The other two cells, P5.p and P7.p, receive less LIN-3/EGF from the anchor cell and also a Notch signal from P6.p. That is why they become the 2° vulval cells. Then, after having started vulval development by selecting the vulval cells, the anchor cells connects the uterus, of which it is a part, to the vulva. The process that

starts the formation of this connection is called anchor cell invasion. It is a very special and rare developmental process, because it involves a cell that crosses tissue borders. In the case of anchor cell invasion, the uterine anchor cell crosses the border, composed of two basal laminae, and enters the vulval tissue. This process is of great interest to us because it is similar to what cancer cells do when they metastasize. In fact, it has been shown that some of the genes that regulate anchor cell invasion also regulate the ability of cancer cells to metastasize. But the anchor cell has even more to do. After invasion, it is required to orient how the 2° cell P7.p develops. It does so by sending a signal called Wnt. Then, the anchor cells instructs the formation of a group of specialized uterine cells. These cells will connect the vulva to the uterus. The signal that selects these uterine cells is again Notch. The last task the anchor cell has during regular development is to free the space through which the eggs will pass. To do so, it fuses with other uterine cells, forming the uterine seam cell in the process. This leaves behind a very thin cell layer that separates the uterus from the vulva. This is the hymen of *C. elegans* and it will be broken when the first egg is laid.

During my studies, I looked at two of all these roles of the anchor cell in more detail. The first part of my studies dealt with the process of anchor cell invasion. I characterized the gene *madd-2*, which is named after another process it regulates called muscle arm extension. It was known when I started the project that the *C. elegans Netrin* gene *unc-6* guides the anchor cell during invasion. If *unc-6* is missing, the anchor cell has no information where to invade and does not invade. What we found when characterizing *madd-2* is that a lack of *madd-2* also stops the anchor cell from invading. But, when we compared the anchor cell between *unc-6* and *madd-2* mutants, we found a big difference in how they looked. During invasion, the anchor cell forms protrusions towards UNC-6 and the vulva. In the absence of UNC-6, the anchor cell tends to not form protrusions at all. Surprisingly, the anchor cell in *madd-2* mutants showed an increased number of protrusions, and not just towards UNC-6. And when we combined mutations in *unc-6* and *madd-2*, the invasion defects did not add up, but were similar to *madd-2* single mutants. Finally, even when both UNC-6 and vulva were absent, we could see that the anchor cell without *madd-2* still formed protrusions. Because of this, we think that the role of *madd-2* during anchor cell invasion is to allow UNC-6 and the vulva to guide invasion and to suppress the formation of unguided protrusions. In theory, this role is extremely important, because of the devastating effects invading cancers can have. Practically, a concrete implication of *madd-2* in metastasis formation or suppression has not been described. Only in one study that correlates invasion with gene expression can such a connection be found. Good data and functional evidence is missing

to strengthen this possible role of *madd-2*.

The second part of my studies was about how the anchor cell secretes LIN-3/EGF when it selects the vulval cells. Vulval induction is a thoroughly studied example of how cells receive their instructions for dividing, growing, etc., also known as their cell fates. As mentioned above, the two main signals used for this are LIN-3/EGF and Notch. It is the anchor cell that produces LIN-3/EGF. The 1° cell P6.p receives most LIN-3/EGF, and its neighbours P5.p and P7.p receive less. This is one reason why we think LIN-3/EGF is secreted and distributed in a graded manner. From studying anchor cell invasion, we know that the anchor cell is a highly polarized cell, most of its proteins and even the lipids in its membrane are not distributed uniformly, but instead concentrated in one place. At some point, we started wondering whether the polarity of the anchor cell plays a role during vulval induction and whether it determines how LIN-3/EGF is distributed. From previous experiments it is known that if the vulva precursor cells are more sensitive to the LIN-3/EGF signal, LIN-3/EGF distribution determines which cells are induced. If LIN-3/EGF reaches other cells than P5.p, P6.p, and P7.p, these other cells then can become vulval cells. Such a case when more than three cells try to become vulval cells is called multivulva. For the worm, this is not beneficial, because the excess cells interfere with proper vulval development. We found that changing anchor cell polarity changes how is distributed. When the vulva precursor cells are more sensitive, this can lead to a multivulva phenotype. If the vulva precursor cells have normal sensitivity, an altered LIN-3/EGF distribution can change which cells become the vulva. Specifically, P4.p, P5.p, and P6.p can form the vulva instead of P5.p, P6.p, and P7.p. This is something that does not happen during normal development, but whether it has any influence on egg-laying is not known. What is important in our finding is that LIN-3/EGF is not distributed at random, but needs to be regulated. How this is done is not clear yet. Generally, signalling is studied in the cells that receive the signal, like the vulva precursor cells. How the signal is produced and sent, however, is being neglected for the most part. We identify the cell sending the signal to be able not just to change signalling but to be important in controlling which fate the signal receiving cells adopt.

The anchor cell is a cell that has many different roles during development. In my thesis, I describe two processes in more detail and add to their understanding. The anchor cell is mostly used as a model for cell invasion today, because it is medically highly relevant. However, the anchor cell does much more than just invade, and it is more than just the cell that produces the signal for vulval induction. The anchor cell can be used to not just study other developmental processes, but also how all these different processes are

executed by a single cell.

4 Abbreviations

AC	Anchor Cell
Cas	CRISPR associated
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
EGF	Epidermal Growth Factor
EMS	Ethyl Methanesulfonate
EMT	Epithelial-mesenchymal transition
GFP	Green Fluorescent Protein
Muv	Multivulva
NICD	Notch Intracellular Domain
PDEF	Prostate Derived Ets transcription Factor
PEDF	Pigment Epithelium-Derived Factor
Pvl	protruding vulva
Ras	Rat sarcoma
RNAi	RNA interference
RPE	Retinal Pigment Epithelium (cell)
RTK	Receptor Tyrosine Kinase
SynMuv	Synthetic Multivulva
TGF-α/β	Transforming Growth Factor α/β
utse	uterine seam-cell
uv	uterine ventral (cell)
VEGF	Vascular Endothelial Growth Factor
VPC	Vulval Precursor Cell

5 Introduction

5.1 A brief historical perspective

5.1.1 From humble beginnings...

It has been 40 years since Sydney Brenner published an article titled "The Genetics of *Caenorhabditis Elegans*" [1]. It has since been cited approximately 8500 times, whereas there are about three quarter million publications referencing *elegans* alone. This article laid the basis for work on a new model organism.

Brenner stated in his seminal introductory paper that he chose *C. elegans* to study behaviour in the context of neurobiology because of its low number of neurons. The undertaking turned out to be more complex than first imagined. Even the relatively simple neuronal network of *C. elegans* is complex and enables sophisticated tasks such as learning. Only in 2012 were individual neurons first manipulated directly, thereby changing the behaviour of *C. elegans* [2]. This kind of research, in my opinion, will lead the way towards a complete understanding of the neuronal network of *C. elegans* and how it controls its behaviour.

5.1.2 ... to a source of innovation

In the beginning the pursuit of neurobiological and behavioural understanding was systematically pursued, especially in the area of movement (there are 111 genes named after the uncoordinated movement phenotype they cause). This lead to the identification of many genes involved in neurogenesis, movement, and cellular trafficking. *C. elegans* turned out to be an extremely potent tool to study many other areas besides neurobiology, especially cell fate specification. It is the nature of *C. elegans* that makes it such a useful model: The simple body plan, invariable development, and easy microscopy allow the identification and comparison of the same cell between wild-type and mutant worms.

Less than 20 years ago, *C. elegans* was the first multicellular organism with its whole genome sequenced. This provided a new opportunity to address biological questions more systematically, especially when combined with RNAi. RNAi was first discovered in *C. elegans* and enables researchers to systematically knock-down any (predicted) gene to test its function [3]. The discovery of RNAi marked the beginning of genome-wide reverse genetic screens, the discovery of new components to old pathways, and new roles of known genes.

A second major innovation partly pioneered in *C. elegans* are fluorescent protein tags, most prominently the green fluorescent protein (GFP). GFP simplifies the study of expression patterns and subcellular localization of most proteins. GFP was discovered in 1962 by Osamu Shimomura [4] and the development of its many modern descendants was spearheaded mainly by Roger Tsien. *C. elegans* was used to first express GFP heterologously and visualize the expression pattern of genes by fusing regulatory sequences to the coding sequence of GFP [5].

5.1.3 *C. elegans* research today and tomorrow

From transgenesis to genome engineering

Since the discovery of the CRISPR/Cas9 mechanism in 2012/2013 it is now possible to modify individual DNA bases in vivo [6, 7]. This tool will transform how research is done because genome engineering has never been this achievable as it is today.

Genome engineering has always been a big part of research. There are two ways to study gene function. The genetic experiments using mutants and epistasis, essentially deducing the function of a gene from its absence, is one way. The other is to use reporter constructs, fusing the regulatory region of the gene of interest to an easily observable reporter gene product to study its expression pattern. The *lacZ* gene, encoding an enzyme that transforms X-gal from colorless to blue, was the reporter gene of choice for a long time. Nowadays, *gfp* or one of its derivatives are the common reporter genes. It is important that the reporter gene expression pattern resembles that of the gene of interest. For that, choosing the right regulatory sequences is central. But choosing correctly is not always trivial.

In *Drosophila*, researchers use transposable elements, pieces of DNA that can jump in and out of the genome, as vehicle to insert artificial DNA into the genome and express it. In *C. elegans* it is much simpler. Injection of pure DNA into the germline of the worms results in the spontaneous formation of semi-stable chromosome-like structures. Any injected DNA can also be expressed from within these extrachromosomal DNA arrays. This is a very convenient method, but also messy and with a number of downsides. The arrays are semi-stable and can be lost, their expression levels fluctuate, and they are not expressed in the germline. Extrachromosomal arrays can be integrated into the genome. This prevents loss and provides stable expression, but still not in the germline. Recently, heterologous DNA is often integrated into the genome with the help of transposable elements also in *C. elegans*. The mariner transposon can be used for the creation of DNA double strand

breaks [8]. This activates the cellular DNA repair machinery, which can be tricked to repair these double strand breaks using a repair template. Designing an artificial repair template with changes to the repaired region can modify the genome itself. This technique is limited by the creation of the double strand break. Recently, an ancient acquired immunity system that can generate DNA double strand breaks was discovered [6]. The system uses two components and can target double strand break creation to virtually the entire genome. This lifts the limitation of creating the double strand breaks and enables pin-point accurate genome engineering. Now genes and their regulation can be studied in their native genomic environment.

The next step in high-throughput research?

Microfluidics, the use of small, PDMS devices that can be designed to fulfill any purpose, is not new. Recently, microfluidics began to be used throughout basic research to scale up and streamline different type of experiments [9]. And now these efforts start to bear fruit. Ideally, microfluidics does not just enable simple high-throughput research, but also high-sensitivity, potentially single cell focussed research. It promises automated microscopy based analysis of many worms sequentially. Additionally, true time-lapse analysis of *C. elegans* development could now be possible, which has so far be hindered by two major factors. Firstly, the necessary immobilization stops the worms from molting at the transitions between the stages, which arrests development. Especially vulval development, which starts in the third larval stage and ends in the fourth, cannot be observed continuously in one single animal. The second hindrance is the way continued immobilization affects the timing and possibly also the outcome of development in *C. elegans*. A worm that does not move is very far from being a wild-type worm. Arguably, this greatly impacts the usefulness of any data collected that way. This does not mean that no useful data can be collected, but the experimental conditions are an obvious deviation from the wild-type. Microfluidics technology could allow the repeated, reversible immobilization of the same worm during its lifetime, allowing it to molt and move normally between observations. The significance and reliability of such data would not be that questionable anymore and really improve all types of high-throughput experiments.

The understanding of *C. elegans* came a long way from its beginnings as a model for behaviour and we might now be on the way to truly understand the control of *C. elegans* behaviour. It did just take a bit longer than Sydney Brenner might have imagined.

5.2 The model organism *Caenorhabditis elegans*

5.2.1 Model organisms

A model organism can be any organism that is studied by researchers. It is called a model organism in the hope that – and because it is generally assumed that – its biology is representative for a broader range of organisms. Different organisms are used to study different questions and processes, depending on the particulars of the organism. Any organism can be a model organism, but some are more popular: *Escherichia coli*, a bacteria, *Saccharomyces cerevisiae*, a yeast, *Arabidopsis thaliana*, a plant, *Drosophila melanogaster*, a fruit fly, *Mus musculus*, a mouse, and *Caenorhabditis elegans*, a nematode. Because we are naturally interested in how we humans function, ideally, we would like to have a model organism as close to humans as possible. Especially to study biomedical questions. None of the aforementioned organisms are very close to humans, although some are reasonably similar. Theoretically, humans themselves would be the ideal model organism. There are also other considerations when choosing a model organism. Besides many practical considerations, which I will allude to below, ethical issues are central. Model organisms are living creatures and as such have dignity. This dignity deserves and needs to be respected. The ethical implications of doing experiments on a living being need to always be kept in mind. Now many model organisms are 'lower creatures', insects, nematodes, weeds, bacteria. As such, they lack certain qualities like a sophisticated neural network and higher cognitive abilities including consciousness. This makes it acceptable to perform experiments that might otherwise be considered unethical. It certainly is not ethical to perform experiments on humans. And that is why we use model organisms and human cells grown in culture to find core principles of biology. With these core principles it will then be possible to infer how our human organism works without the need to directly experiment on it.

All these organisms are studied to find the principles of life. Based on the theory of evolution, there is a common ancestor to life. All current forms of life derive from this ancestor. This is the basis for assuming that core principles of life exist. By studying different species across all different branches of the tree of life, we hope to find these core principles that will also apply to our organism. Even though human cells can be directly studied in culture, the complexity of multicellularity and the organismic context cannot. To understand this, we need model organisms.

5.2.2 The features of a good model organism

A model organism should be a model – a prototype – and representative of many other organisms. This is not necessarily always the case. Most basic characteristics of cell and molecular biology and even cell-cell communication seem fairly universal and similar when compared between *C. elegans*, *Drosophila*, mouse, and human cell culture. How these mechanisms form an organism is usually less likely to conform to an overarching principle. All organisms occupy their own evolutionary niche requiring various adaptations, and slight differences between organisms are thus expected.

As mentioned, a model should be a prototype for the aspect of life under study. No model organism will be a good universal model, because there is no universal species and every species is different. Importantly though not in everything. The chosen model organism should be a prototype for the studied aspect. This is easier said than done, because the specifics of an organism and how much of a model it will be cannot be known beforehand. To determine whether an organism is a good model, it is important to know what it should be modelling, to know what is representative and what is not. Besides being a faithful model, the goal and focus of the research matters. For biomedical research, a high degree of similarity to humans might be beneficial. But studying distantly related organisms could offer new insights and novel cures.

The most influential factor in choosing a model organisms is practicality: Ease of cultivation under standardized, laboratory conditions, speed of growth, space requirements, and financial considerations are factors contributing to whether an organism is a good model or not.

In conclusion, some organisms are good models for certain aspects and other organisms for different aspects. It is not possible in most cases to know in advance which ones are which. But even when models turn out to be bad, they offer insight into evolution and the composition of the tree of life.

5.2.3 The characteristics of *C. elegans* as a model

C. elegans has been one of the more successful model systems. Despite it being around for 60 years less than the arguably most famous model system, *Drosophila melanogaster*, both are important today in their own way, each defining its specialized fields of research. Besides research into genetic pathways and the molecular basis of organ development, *C. elegans* serves as model for aging research, neurobiology, even behaviour and learning, and evolutionary studies [10, ?, 11, ?, 12, ?, 13, 14]. *C. elegans* was chosen as model system specifically because of its relative simplicity, in the hope it

would be especially suited to study its neurobiology and nervous system in particular. More than just the success in for example identifying various components of axon guidance, the popularity of *C. elegans* most likely stems from it just being a very handy model organism. It has many practical advantages over *Drosophila*. First and foremost stock keeping, or maintaining mutations, is so much easier in *C. elegans*. Its hermaphroditic nature usually makes complex, balanced strains unnecessary and long term storage is easy because *C. elegans* can be frozen. The generation time is short for a multicellular organism, and its transparency is great for microscopic observation. These advantages, more than anything else, made the simple nematode *Caenorhabditis elegans* a very popular model organism.

The main thing about *C. elegans* that stands out when compared to other model systems, and organisms in general, is its fixed cell lineage. All of the 959 somatic cells of an adult hermaphrodite have the same exact ancestry in every wild-type worm. They are born from the same cell division every time and they have the same fate every time. They divide or differentiate the same way every time. Or they die every time. And all the divisions, differentiations, deaths of each somatic cell have been mapped.

It is this property that makes *C. elegans* well suited to precisely study cell fate acquisition and cell death. For example, apoptosis could for the first time be studied in detail [15]. Apoptosis is the kind of cell death that is regulated and benign, as opposed to necrosis, which happens in response to defects or infections and is generally harmful. Because of the fixed cell lineage, the timepoints of apoptosis for different cells could be determined. Consequently, geneticists did mutagenesis screens that identified genes that modified the invariable pattern of apoptosis and thus regulate cell death. This meant a big step forward for the field of apoptosis research.

With the age of modern genetics and genome engineering however, even though these advantages are still nice, they are not as crucial anymore. It is now not only possible to transiently knock-down gene expression via RNA interference – which is a well established technique by now – but also to alter virtually every single base in the *C. elegans* genome (and in that of most other organisms) [16, 17]. This is the step that will drive future discoveries in the field of biology.

5.2.4 The developmental stages of *C. elegans*

The mature oocyte is fertilized by the spermatheca before entering the uterus. The embryo will be passed through the vulva to the outside where it continues maturing for nine hours (Fig. 1). After that, a larva will hatch from the egg. It is the first of four larval stages that *C. elegans* passes through under

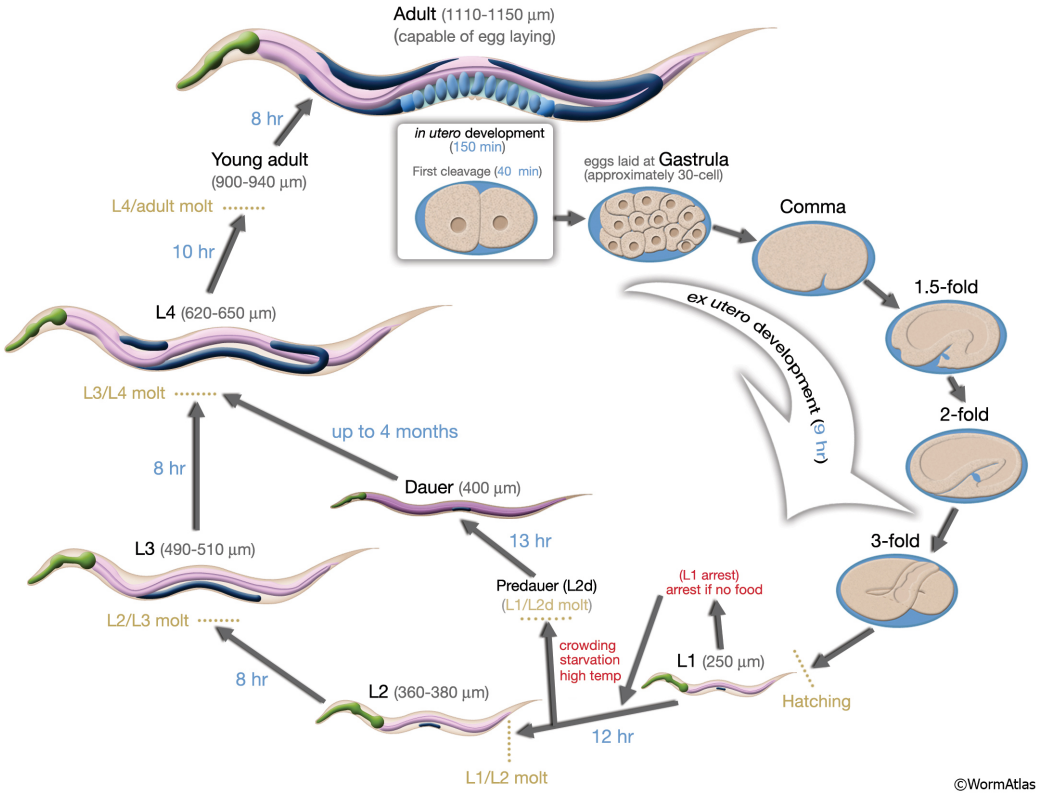

Figure 1: The *C. elegans* life cycle
The life cycle of *C. elegans* at 22°C. It shows the development from the fertilized zygote to the adult hermaphrodite with approximate timings. The alternative developmental dauer stage is also shown. The illustration is taken from wormatlas.org

non-starved conditions before reaching adulthood. The first larval stage lasts for about twelve hours followed by the first molt and beginning of the second larval stage. In this stage, the somatic gonad starts proliferating and the anchor cell is specified. The following third larval stage is when vulval development starts. The vulval cells divide and the barrier between vulva and uterus is broken during anchor cell invasion. It is the stage most relevant to the research I carried out. Then comes the fourth and last larval stage. During this stage, *C. elegans* hermaphrodites produce sperm before they switch to producing egg cells. Vulval development continues and is concluded by morphogenesis. Also, the connection to the uterus is fully established and the somatic gonad formed. At the end of this larval stage, the vulva everts and becomes a fully functional egg-laying organ.

5.2.5 The relationship between *C. elegans* and humans

In the end, research is not just done to satisfy curiosity, but the interest generally lies in trying to understand our own organism better. Model organisms essentially serve to emulate certain aspects of the human organism and development. Superficially, *C. elegans* has not much in common with humans. It is a hermaphroditic worm species, whose main organs are gut and gonad/vulva. It has a fixed cell lineage and several larval stages. Its immune system is primitive and no cancer development was ever observed in *C. elegans*. Despite the huge, organismic differences, on a molecular level, worms are surprisingly similar to humans. Many pathways that are crucial for basic cellular processes, such as cell growth, cell-cell communication and adherence are conserved at the core. And even more complex processes like cell invasion do have closely related molecular components within the two organisms. What that means is that *C. elegans* can in fact be a useful model to understand certain aspects of human physiology and especially cellular processes. On the other hand there are more complex fields, such as neurobiology, behaviour, learning and processes involving different groups consisting of multiple cells each. When trying to find equivalents for these fields, a very simple organisms like *C. elegans* with comparatively few cells can prove useful in parts, but will never enable us to understand the details.

5.3 Why study a worm?

5.3.1 Men and nematode – not as different as could be thought

It might seem odd to do research on a little worm that shares seemingly few traits with us humans. It has a fixed cell lineage, humans do not. It can exist as asexual hermaphrodite, humans can't. It's an invertebrate, humans are evolutionary distant mammals. Yet despite all the differences, there are important commonalities too. Not at the macroscopic or microscopic, but at the molecular level. Much of the cellular architecture and many genetic pathways are very similar compared between the tiny worm *C. elegans* and us humans. Conservation actually allows findings of how pathways work to be directly transferred from worms to men. An example of this is the aforementioned guidance molecule UNC-6/Netrin. UNC-6/Netrin was discovered as *unc-6* in *C. elegans* first [18] and later rediscovered to stimulate the outgrowth of rat neurons *in vitro* [19]. Similarly, many other genetic pathways, such as Notch, Wnt, or the RTK/Ras pathway, all of them crucial for the development of higher organisms including humans, are highly conserved and are studied in *C. elegans*.

5.3.2 Cancer – a disease with evolutionary ancient characteristics

Cancer is a deadly disease and overall the second most common cause for mortality worldwide, responsible for just under 20% of deaths among the 30 to 70 year old people [20]. The most common cause are cardiovascular diseases and diabetes with over 30% of deaths. But cancer is the much more prominent disease. Cancer actually is not just one disease, but a collective term for many different diseases. This is certainly part of the reason why a cancer cure is difficult to find. There is no "one fits all" remedy. Despite cancer being many different diseases grouped together, the grouping makes sense. Cancer can basically be characterized as cells reverting back to a primitive, quasi unicellular, state. What does that mean? Multicellular organisms are fundamentally different from the evolutionarily basal single cell organisms. In single cell organisms, survival of the organism is equal to survival of the individual cell. In multicellular organisms, the vast majority of cells, the somatic cells, will not transmit their genetic information directly to the next generation. The germ cells, eggs and sperms, are those cells of a body that actually pass their DNA to the next generation and are evolutionarily successful. All the somatic cells of a body serve the purpose of propagating the germ cells and put the survival of the germ cells above their own survival. Cancerous cells do not have this particular, evolved behaviour anymore.

Cancer research is done in *C. elegans* with success. This might seem surprising because *C. elegans* do not develop cancer. Importantly though, as mentioned earlier, genetic pathways are conserved between *C. elegans* and humans. This is very well exemplified by the mechanism of AC invasion, which form the first part of this thesis. David Matus *et al.* managed to demonstrate that how human melanoma cells breach tissue borders is very similar to how the AC invades [21].

5.4 Genetics

5.4.1 What are genes and what is genetics

Genes are the hereditary unit, a segment of DNA that controls the expression and production of a gene product, typically a chain of amino acids, aka a protein. Genes are believed to control to a large degree what and who we are. The genome, the completeness of all genes, thus has become a focus of research as of late, driven to a large degree by new technology enabling DNA sequencing *en masse*. Indeed the cause of many diseases can be found in one or more of our genes.

Another definition of genome can refer to the complete genetic material, or DNA, in our cells. The difference between the two definitions is big. Genes are defined as functional units, whereas far from all genetic material or DNA does have a clear, evolutionarily important function. Traditionally, more than 90% of our DNA was considered junk, without function. A recent survey however, the ENCODE project, claimed that 80% of the genome has some form of function, and be it only binding of a certain factor [22]. However, the idea that simple protein-DNA interaction removes its junk status has been criticized [23, 24].

Genetics is the study of genes. Possibly the first geneticist was Gregor Mendel, a bean-counting monk who determined a hereditary pattern of certain bean phenotypes. At that point, a gene was nothing but a concept. Only much later could it be shown that these hereditary units are linked to chromosomes and DNA by Barbara McClintock. This revelation and the emergence of techniques allowing the manipulation of DNA changed the work of a geneticist profoundly.Today's geneticists are not just interested in what a gene does, but what the individual segments of a gene do, how a gene is regulated and with which other genes it interacts in a given biological process.

5.4.2 How genes are studied

Along with the discovery of what a gene is physically, what the chemistry of DNA is and how it can be manipulated, came a change in how the function of a gene is being studied. Traditionally, genetic screens were the way to identify new genes. Screen refers to the basic element of the whole process, which is to screen many, many individuals (bacteria, animals, plants) and to look for a new phenotype. It most commonly involves treating the individuals in question with a mutagen. In that way, it is possible to identify mutants that are important for a specific process, judged according to the mutant phenotype. Notably though, the phenotype is not linked to a specific DNA segment, and it is traditionally a lot of work to identify the mutated gene. With the advent of quick whole genome sequencing, made possible by high-throughput methods, the process became easier. Today a gene is almost always a protein blueprint, but in principle a gene can be anything. In fact the physical representation of a gene does not have to be known to do a geneticists work. Nowadays, a lot of work focuses on the molecular nature of the gene under study. The classical approach of building double and triple mutants to study the place a gene in a pathway, though still important, tends to be more and more marginalized. Instead, whole genome and transcriptome approaches are used to try doing classical genetic work in a more effective way. This also includes the study of graded phenotypes

in quantitative genetics and the association of one or more phenotypes with the whole genome in genome-wide association studies. These techniques can assess a percentage contribution of a certain gene or locus to a specific phenotype. And whilst these approaches do yield results, the nature of these results are qualitatively different compared to the traditional, single gene focussed approach. Nevertheless, the basic methodology is the same. The goal is to deduce the function of the gene in question, typically by identifying defects in mutants with either single or a multitude altered genes. Whilst this approach might seem counter-intuitive, it has proven very powerful and successful. It is the easiest way of trying to understand what is happening inside a black box. Grouping genes according to phenotypes creates functional units, e.g. genetic pathways. By looking for enhanced or suppressed phenotypes, more genes can be added to such pathways and a hierarchy can be inferred.

5.4.3 The genetics of *Caenorhabditis elegans*

The genetic makeup of *C. elegans* is relatively simple. It has five sets of autosomes and one set of sex-chromosomes. XX animals develop as sexual, self-fertilizing hermaphrodites, X0 animals as males. Because of self-fertilization, males are rare in laboratory populations. Males can arise spontaneously due to meiotic non-disjunction events. Hermaphrodites produce around 300 sperm cells during the fourth and last larval stage. Adult hermaphrodites produce egg cells until the end of their life. It is a small oddity of *C. elegans* that usually the sperm - and not the egg - limits the number of progeny. The hermaphrodites of *C. elegans* are sexual however and can mate with males, which increases the number of potential offspring.

The *C. elegans* hermaphroditic lifestyle is tremendously advantageous for genetics and stock-keeping. When doing crosses, once all the desired alleles are present in the same animal, waiting and continuously testing individual worms will yield the desired outcome. Similarly, once a hermaphrodite is homozygous for an allele, that locus remains homozygous. Males can be ignored.

On the downside, *C. elegans* does not have the same amount of sophisticated tools as *Drosophila* does. But *Drosophila* genetics is also around 80 years older. Genetic balancers are probably lacking the least, although even this tool-set is incomplete. Also Gal4-UAS-like and FLP/FRT systems for modular gene expression and genomic recombination start being developed, but they are still far from forming a comprehensive tool-kit.

Newer tools, developed within the last five years, largely overcome these drawbacks. Specifically, the availability of targeted genome engineering using

the Mariner transposon and the CRISPR/Cas9 system are the genetic toolset of the future [8, 7].

5.4.4 Genetic screens

A genetic screen is a high-throughput method to identify genes that regulate a certain process. Typically, identification relies on a specific phenotype, usually appearance or behaviour, but it can be anything from gene expression to antibiotic resistance to protein localization. Most of the times, a screen is done after inducing random mutations in the genome of the studied organism. Numerous different methods and agents can be used for mutagenesis. Traditionally, chemical screens are most common, using ethyl methanesulfonate (EMS), a substance that leads to thymine nucleotides replacing cytosines, as the mutagen. Ideally, every single gene is mutated and screened individually. Once the organism has been mutagenized, many thousand individuals of the next generation are screened for the phenotype of choice. This is followed by isolation of the mutation and experiments to determine how the mutated gene regulates the studied process. A screen really is only a first step to identify potential regulators, and any newly identified gene needs to be tested and its specific function and position in a genetic pathway identified.

The two main methods of identifying new genes are forward genetics and reverse genetics. As can be gathered from the names, these two approaches differ quite extensively, even though the underlying principle of screening remains. Forward genetics starts exploring the function of a gene at the level of the phenotype and eventually identifies its molecular nature. Random mutagenesis is the method used for forward genetic screens. Reverse genetics is possible only since the discovery of RNAi and the availability of whole genome sequences. It aims to identify the phenotype and function of a gene starting from its base pair sequence.

5.4.5 Forward genetics

Early work in model organisms such as yeast, *Drosophila* or *C. elegans* has identified many important principles of development and conserved signalling pathways. As mentioned above, the method of discovery was relatively simple. Random mutagenesis and subsequent isolation of phenotypes. But despite the obvious power and success of the forward genetic approach, there are downsides.

First, random mutagenesis is in fact pseudo-random mutagenesis. Not all parts of the genome are equally accessible to mutagens. And not all parts of the genome can be mutagenized by every mutagen. There are always certain

hotspots where the chance of a mutation is higher and coldspots where mutations are less likely to occur or do not occur at all. Consequently, in order to screen all genes, the number of genomes that need to be screened is disproportionately high. Additionally, random mutations have a higher chance of being disruptive rather than creative; genes are more often knocked-out then activated. This makes the identification of active genes more likely, because their knock-out tends to cause a bigger change and a more pronounced mutant phenotype. Another downside of random mutagenesis is the chance of having multiple mutated genes in the same individual. This can obscure the phenotype and make the identification of single genes much more difficult. Two factors are thus crucial for mutagenesis: The concentration of the mutagen, which controls how frequent mutations are, and how many individual organisms are screened, which determines the likelihood of testing all the genes.

Second, forward genetics relies on the identification of phenotypes after mutagenesis. For some phenotypes, this is simple. For others, it is more complex and time-consuming, but still doable. Finally there are phenotypes that are either too subtle or too complex to detect and will be missed. There are tricks to make such phenotypes more detectable, for example by using sensitized genetic backgrounds, but this is not always feasible. The result is genes not discovered by forward genetics.

In conclusion, forward genetics is a powerful and successful method to identify and characterize most, but notably not all, genes.

5.4.6 Reverse genetics

Forward genetics identifies phenotypes and then finds the associated gene, reverse genetics targets the gene, knocks it out, and then checks for phenotypes. Reverse genetics is thus not suited discovering new genes, and less suited to discover novel phenotypes. This is mostly due to the effort required for setting up a genome-wide reverse genetic screen, which requires all the genes to be knocked-out and analysed individually. Thus for every gene, a population of organisms has to be tested, whereas in a forward genetic set-up, a single population can contain mutations in every gene.

The reverse genetic approach is possible because of new discoveries and the development of new technologies. The genome sequence (and the sequence of every gene) must be known and RNAi had to be discovered. RNA interference (RNAi) is a process inherent to most organisms we know and which cells use to protect themselves against viral infections. It does so by cleaving double-stranded RNA. Crucially, it can be targeted to cleave specific, endogenous mRNAs, thereby knocking-down a specific gene product.

Theoretically, this can turn off any gene. Practically, there are a few restrictions. Firstly, the sequence of a gene must be known in order for it to be knocked-down. Secondly, RNAi based gene knock-down is not always efficient and hardly ever complete. Usually, low level gene activity remains. For some genes, RNAi knock-down is completely inefficient. Newer technologies, e.g. Mos or CRISPR/Cas9, can offer an alternative in these situations. However, the huge advantage of RNAi is the absolute ease with which genes can be knocked-down. RNAi works systemically in *C. elegans*, which means that gene knock-down is not restricted to individual cells or tissues and the RNAi effect propagates throughout the organism. Because of that, genes can be knocked-down organism-wide by simply feeding *C. elegans* with bacteria expressing double-stranded RNA. This easy setup makes whole genome reverse genetic RNAi screens relatively quick and affordable. They also offer an attempt at combining the systematic examination of every gene typical of reverse genetics with the unbiased examination of forward genetics. This attempt is however limited by the design of the RNAi clones and the inability of RNAi to alter genes in other ways than reducing their function. Other reverse genetic tools are much more laborious and not as suitable for whole genome approaches, but instead used for targeted, single gene applications only.

5.4.7 Sensitized screens

Knocking-down or mutagenising genes in a wild-type genetic background will mostly reveal positive regulators. Genes that are negative regulators are unlikely to be identified in this manner, which is an obvious drawback. To overcome this restriction, genetic screens are done in a mutant background instead of wild-type. The underlying principle is relatively simple: In a wild-type situation, knocking-out negative regulators increases pathway activity. In many cases, this will not affect the phenotype – if a signal is there, the strength of the signal is less important. An increase in pathway activity in a situation where the signal is missing or low, an increase will likely revert the mutant phenotype to wild-type. And this can be easily screened for. This approach relies on pre-existing pathway mutants and restricts screening to one pathway and/or one phenotype at a time. Alternatively, target gene expression levels could be monitored directly to identify negative regulators. Albeit possible, such experiments are laborious and expensive.

In conclusion, although sensitized screens are by design constricted, they are a powerful tool to uncover negative regulators.

5.4.8 Conclusion

Genetic screens form the basis for our understanding of molecular biology. They unravel the mechanisms and pathways working inside cells and of organisms alike. However, as I tried to point out, no screening approach, neither forward nor reverse, is perfect. Genes can be overlooked and phenotypes ignored. One approach alone will not uncover the complete picture. It is the combination of the two, forward and reverse genetic screens, that might show us a more comprehensive picture of the genes and their function in a specific process.

It was with this rationale in mind that a former PhD student in the lab, Dr. Peter Gutierrez, did his thesis. He, as I in the second part of my thesis, looked at interactions with *gap-1*, trying to find genes regulating LIN-3/EGF signalling and LET-23 localization, based on the findings by Alex Hajnal [25]. It is in fact the reverse genetic screen he started that I completed. And as we hoped, we found a novel mechanism important during vulval induction. Without the reverse genetic approach, we probably would not have found it.

Even though combining forward and reverse genetic screens might identify additional genes and offer a more complete understanding of genetic pathways and networks, even this understand is, in my opinion, still far from true comprehensiveness. Partly this is due to the nature of the methods. We are limited by pseudo-random mutagenesis and variable RNAi effects. But an even bigger obstacle is the strength of possible phenotypes. Genes that balance and fine-tune pathway activity, genes that regulate only a few downstream targets of a pathway, and emergency contingency mechanisms will be hard to identify because of their functions. Many different sensitized screens will be required to uncover and describe every genetic pathway comprehensively.

5.5 Nature *versus* Nurture

The nature *versus* nurture debate deals with the influence of genes on who and what we are and how big the influence coming from other sources is. For a long time, any trait we have was thought to be determined by the composition of our gene pool. During maybe the last decade, the all-determining role of the genes has been called into question. The discovery of epigenetic marks certainly was a major contributor to this. Epigenetics is probably the most studied mechanisms emphasizing environmental factors in determining who we are. But even before the discovery of epigenetics, twin studies never could fully attribute our phenotype to the genome alone.

Epigenetics influences who we are not by manipulating the genetic code

(the order of the nucleotides in our genes), but by modifying our genome in a different manner. So-called epigenetic marks are varied, semi-stable modifications of the phosphate backbone of the DNA. These marks can be inherited but also changed by a number of different enzymes. Whereas the availability or lack of resources and nutrients has an obvious effect on the development of the current generation, it also changes the makeup of our epigenetic code and through it the developmental program of coming generations [26].

What do epigenetic marks do? The various different marks on both DNA and its packaging proteins, the histones, control how and when a gene is expressed. Their importance highlights how crucial precise control of both spatial and temporal gene expression is. Notably, this control does not depend on the sequence of the nucleotide and only involves non-coding information. However, any purely structural and/or histone based regulatory elements are far less specific than those based on a unique nucleotide sequence. The quasi-ubiquitous substrate for epigenetic marks, the backbone of the DNA and its histones, suggest that any alteration of the epigenetic machinery would have a gross effect on gene expression. The observation that it does not however, highlights our immense lack of understanding of these non-genetic regulatory mechanisms.

What brings this nature versus nurture argument full circle is the fact that placing and removing the various epigenetic marks is done by proteins. And these proteins are expressed from genes. Obviously, only active genes produce the modifying proteins that can place and/or remove epigenetic marks. Also, some germline specific epigenetic remodelling removes whole sets of epigenetic marks and places others. The most extreme example of such non-regulated epigenetic regulation is the Barr body. The Barr body is the condensed X chromosomes in females. It is the result of complete transcriptional inactivation of one, seemingly randomly chosen X chromosome in every cell.

We think that the genes and the genome forms the core of what we are, however, not everything is hardcoded into the genetic code. Regulatory mechanisms are manyfold and can act on a level above the primary nucleotide sequence. Marks can be placed on DNA and proteins without changing the primary sequence and this can heavily influence how any gene or protein operates. We are starting to understand which proteins regulate which marks and how each mark tends to influence gene expression, but how genome wide regulation works and how global changes in modifying enzymes can only exert a local influence is still unknown.

5.6 The wild-type genotype and laboratory strains

In order to test any process, pathway or protein activity, the results need to be compared between the mutant strain in question and the wild-type. Wild-type generally is the reference strain N2, a strain not supposed to carry mutations that are not also found in free living *C. elegans*. However, the N2 strain was isolated more than 60 years ago and has been in culture ever since and most likely propagated for at least 20 years. What does that mean? The average generation time of fed *C. elegans* is roughly four days and when starved, the lifetime of one generation can stretch to around four weeks. Also, *C. elegans* can be frozen which basically stops the evolutionary clock. Even so, the N2 wild-type strain has spent a considerable amount of time, potentially up to between 300 and 2'000 or more generations in the laboratory . Importantly, it is kept under stable and controlled conditions that are optimal for growth. If we assume an average 20 years for human generations, that would correspond to between 6'000 and 40'000 years of sheltered human development with no evolutionary pressure. The top end estimate corresponds to the time passed since *Homo neanderthalensis* became extinct. Not too surprisingly, also considering that a worm is a simpler system with less functional constraints, many mutations can accumulate. And they did. By comparing our wild-type strain N2 to wild isolates cultivated only recently, many differences can be found [27]. And the phenotypic differences alone between N2 and a relatively old isolate commonly referred to as 'Hawaii' are already big. N2 worms like to spread out on a plate, Hawaii worms clump together; N2 worms do not burrow into the agar of the plates, Hawaii worms do; the male frequency in N2 is very low, in Hawaii it is common to find spontaneously generated males; Hawaii males plug the hermaphrodite's vulva after mating, N2 males do not; etc. Most of the changes in N2 could in fact be considered adaptive to lab conditions, simplifying handling and increasing fertility. And yet, we refer to both strains as wild-type. And even though this term is used relatively and to indicate the absence of known mutations, distinguishing the two different genetic backgrounds can become tricky depending on the experiment. Theoretically, these changes do not matter too much because all the generated mutants are based on this N2 strain. This means that any mutant differs from the N2 strain only in the mutated gene and N2 can be declared as wild-type. Practically, there are many different labs worldwide that work with *C. elegans*, and they all use the N2 wild-type strain as reference. But, in the same way that the N2 strain differs from wild isolates, the various N2 lab strains differ from each other. Most likely not to that heavily, but they do. And different N2 populations within a lab can differ from each other. Naturally one would assume that even

though there are mutations, they probably do not affect major developmental processes. But they do. Most likely, N2 and any other established lab strain is so domesticated that survival true wild-type conditions is not possible.

Another factor that leads to mutations being accumulated in the lab is the fact that we work with mutants. Many mutations can be and are kept homozygously, even when affecting development. This decreases the evolutionary fitness of these strains, which means that their reproduction is less than optimal. Consequently, any spontaneous mutation that increases fertility and number of progeny is selected for. And these mutations occur on a notable scale, judging by experience. Importantly, added mutations do not change the original one, but occur hidden. Usually, we monitor the original mutation on a molecular level, and do not characterize other phenotype like progeny number. It is thus very difficult to notice freshly acquired mutations and to judge whether a mutant has a 'pure' N2 wild-type background or whether there are additional mutations.

If a new mutation occurs in an already mutant background, it should not spread further than the strain it happens in. But if the same happens in the N2 strain, it would still be used as reference strain for experiments, starting point for crosses, and mutageneses. New mutations, that do not necessarily change any phenotype significantly, will accumulate. This potentially happens in all N2 populations, but not exactly identically, and then the wild-type will eventually differ from the wild-type.

What is the conclusion from this? To be care- and mindful when using the expression wild-type, for experiments to ideally include the progeny from the same cross and not just any wild-type worms in the analysis. And especially when working with subtle phenotypes, it is worth keeping in mind that there is a lot of variation within the genetic background of *C. elegans*. Essentially, we do not know how strongly the genetic background can impact even the basal level of the processes we study.

5.7 The Anchor Cell

5.7.1 Anchor Cell specification

The lineage identity of the anchor cell (short AC) can either be Z1.ppp or Z4.aaa and part of the somatic gonad. It is born in the beginning of the second larval stage. Lateral inhibition via LIN-12/Notch signalling specifies either Z1.ppp or Z4.aaa to be the AC [28, 29]. Both prospective ACs express ligand and receptor to signal reciprocally. Activated receptor reduces ligand expression. The other cell thus receives less signal and is not inhibited from producing ligand itself. This system amplifies small differences in ligand

concentration between the two cells. Finally, the cell expressing more ligand adopts the anchor cell fate. Which cell becomes anchor cell is mostly random, but the age and *hlh-2* expression levels in the precursors are contributing factors influencing the decision [30].

5.7.2 Anchor Cell development

The AC is involved in different developmental processes: AC specification via LIN-12/Notch signalling, vulval induction by the AC through LIN-3/EGF secretion, AC invasion guided by UNC-6/Netrin, uterine π fate specification, again through LIN-12/Notch signalling, and finally dorsal lumen patterning and morphogenesis, during which the LIN-3/EGF signal likely plays an instructive role [28, **?**, 29, **?**, 31, 32, 33, **?**]. The AC is a cell central to the development of the vulva and the uterus and has many different roles throughout development. This makes it an interesting model to study, because many different processes can be studied in the same model and because the failure of any of these processes gives a clear, easily identifiable phenotype without compromising viability. It is of note that as development completes, the AC loses its function and ceases to exist by fusing to a number of other uterine cells.

Despite the various roles of the AC and its continued, changing activity, an overarching understanding of the AC's life is missing. AC specification is partly understood and mainly influenced by a combination of time of birth relative to its competitor and *hlh-2* expression levels. *hlh-2* promotes *lag-2* expression and later also LIN-3/EGF production. *hlh-2* seems crucial in the first half of the AC's lifetime to fulfill its role in specification and vulval induction. *hlh-2* might be important during uterine patterning through its targets *lag-2* and LIN-12/Notch still. Recently, *hlh-2* function has been linked to AC invasion, too [34]. I find it difficult though to see this as a novel mechanism and not an extension of incomplete AC specification due to loss of *hlh-2* [35]. Thus, whether or not *hlh-2* really is the factor that defines the role of the AC throughout its lifetime is not clear in my opinion. For AC invasion, but not for guidance though, *fos-1* is the master regulator. *fos-1* also positively regulates *hlh-2* [34]. This might suggest a more central role for *fos-1* than for *hlh-2* overall. An involvement of *fos-1* in AC specification however is not documented and *fos-1* mutants do not show AC specification defects. But the interaction between *fos-1* and *hlh-2* might suggest a feedback loop and a connection between on the one hand AC specification, vulval and uterine induction and AC invasion on the other. These processes are not connected to AC guidance on a molecular level still. Interestingly, the AC's final task of fusing with the utse is mediated by *aff-1* which is again regulated

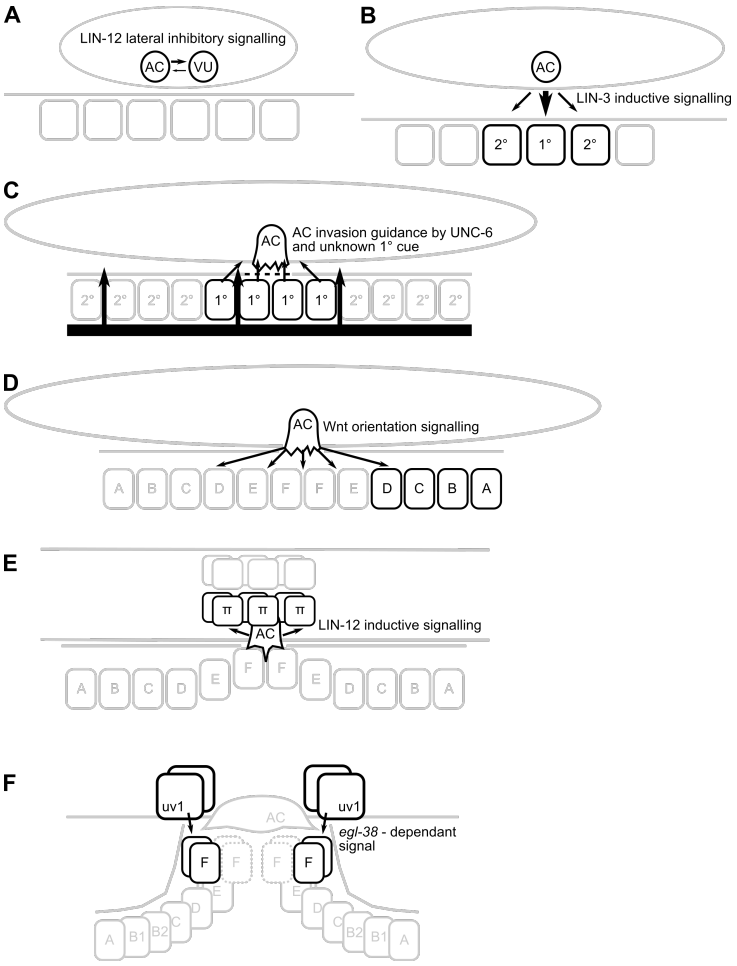

Figure 2: An overview of vulval and gonadal AC development
A schematic representation of the different developmental processes the AC is involved in. The involved components are black, the rest grey. (A) shows AC specification via lateral inhibitory LIN-12/Notch signalling. (B) shows vulval induction via LIN-3/EGF. In wild-type, LIN-3/EGF reaches mostly P6.p. (C) shows the processes of AC invasion and the two main guidance signals. (D) illustrates that the AC orients the 2° cell lineage of P7.p via Wnt. (E) is the last signalling event of the AC, the induction of the uterine π cell fate via LIN-12/Notch. The fusing of the AC to form the uterine seam cell is not shown. (F) shows the last step in forming the vulva before eversion, the formation of the dorsal lumen by *egl-38*-dependant signalling from the uv1 cells.

by *fos-1* [36]. It seems the only process not part of a bigger scheme is in fact guidance. Nevertheless, an actual connection between all the processes the AC is involved in, and not just the hypothesis of one, has not been experimentally demonstrated. Even if a master regulatory connection is found, the temporal regulation of the processes would be of great interest. For example, the role of the cell cycle has never been clarified, possibly because the AC is a quiescent cell without an active cell cycle.

5.7.3 Vulval induction by the Anchor Cell

The AC starts vulval induction. It does so by secreting the growth factor LIN-3, a homologue of the mammalian epithelial growth factor EGF [37, 29]. Classically, LIN-3/EGF distribution is graded along a group of cells called vulval precursor cells (VPCs). These cells are all equally competent to adopt a vulval cell fate. The gradient is interpreted by these VPCs and if the level of received LIN-3/EGF is high enough they become vulval cells.

5.7.4 Anchor Cell polarity and invasion

An egg-laying organ that is not connected to the germline and the uterus is of little use. A crucial part of vulval development is the formation of the uterine connection. Connecting one organ to another is a tightly regulated process that involves breaching tissue borders. It is tightly regulated because violating tissue integrity can be perilous for the organism. In humans, malignant, metastasis forming tumors are such a dreaded disease exactly because these tumors can cross tissue borders.

After the AC starts vulval development, it enables egg-laying by creating a connection between uterus and vulva. Uterus and vulva are separated by two layers of basal lamina, one surrounding each tissue. These basal laminae demarcate the tissue borders and preserve tissue integrity. The AC dissolves these borders and opens them for the future passage of embryos. This process, generally referred to as AC invasion, starts at the beginning of the third larval stage. Signs of tissue breaching are only visible in mid-L3 stage however, halfway through vulval development.

AC invasion consists of two parts. The mechanical and chemical breaching of the barrier, and the knowledge of where to breach. Two signals regulate the guidance. One provides a general direction towards the ventral side and the other towards the 1° vulval cells specifically.

The transcription factor *fos-1* is at the center of the gene network that regulates basal lamina breaching [38]. The AC expresses the *fos-1a* isoform from the start of the third larval stage and *fos-1a* mutants are severely inva-

sion compromised. No other gene is as singularly important. *fos-1* activates *egl-43*, a transcription factor important for AC invasion and the specification of uterine cell fates [39]. *egl-43* activates both *cdh-3* and *zmp-1*, two genes directly involved in basal lamina breaching [38]. *him-4*, another *fos-1* target, is the effector gene with the biggest impact on invasion. Many effectors of AC invasion are still unknown, because 75% of effector triple mutants (*cdh-3*, *zmp-1*, *him-4*) invade successfully (Fig.3). Identification of the unknown components will be difficult, because most of them will only have marginal effects.

The second part of the AC invasion process, as mentioned above, is guidance. The best characterized aspect of this is *unc-6/Netrin* signalling [31]. Axon migration both in mammals and in *C. elegans* is guided by *unc-6*. *unc-6* can be attractant and repellent, dependending on the receptor. The main attractive receptor of *unc-6* is *unc-40*. *unc-5* is a co-receptor of *unc-40* and makes *unc-6* act as repellent [40]. During AC invasion, *unc-5* has no characterized role. *madd-2* is also part of the *unc-6* guidance pathway and is the first part of this thesis.

Another signal, coming from the 1° vulval cells, guides the AC towards the vulva directly. *unc-6* reportedly is a more general directional cue, although *unc-6* too is expressed in the 1° vulval cells [41]. And despite more than ten years of research on AC invasion, the second AC guidance cue still is not identified.

5.7.5 The missing Anchor Cell invasion cue

That the second AC guidance cue is not known is uncommon and unexpected, especially because technology and advanced experimental designs are very powerful and allow finding the answers to any questions. Despite even the knowledge of where the second cue is produced, it remains unknown. There are at least two possible explanations for this. One, the cue is in fact not one, but several signals that contribute in parallel to AC guidance. Then, redundancy is the problem, which is difficult to deal with and takes more time to overcome. Possibly until after the next technical revolution. Redundancy can also be functional in nature, and this is the second explanation. A functional redundant guidance cue would be required during earlier embryonic stages. If the cue is missing, development would not progress and any AC invasion defect be hidden. In this case, cell and/or stage specific gene knock-out would reveal the second guidance signal. Such an experiment is technically possible, for example by using tissue-specific RNAi. These kind of experiments are not trivial, timing and RNAi efficiency need to be tested. Alternatively, mitotic recombinations using the FLP/FRT system could cre-

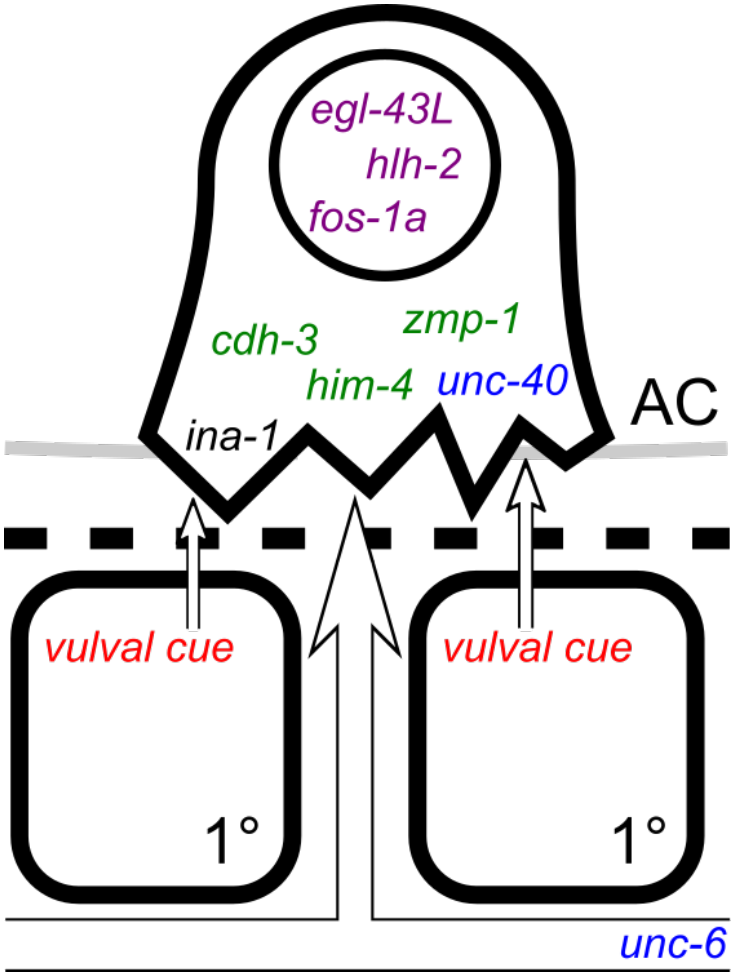

Figure 3: AC invasion
A schematic representation of AC invasion at the mid-to-late L3 stage, including some of the genes involved. Colour and position group the genes according to function.

ate homozygous mutants in the vulval tissue only. But this technique is not fully established in *C. elegans* yet.

In conclusion, the missing guidance cue illustrates that redundancy is very difficult to deal with in biology. Especially when there is very little information about the nature of the specific redundancy. And the reason the cue is still unknown might not even be redundancy. The reason could be completely novel, in which case all the experiments designed to find it would not have been the right ones. And even though this is rather unlikely, molecular biology is still full of surprising findings.

5.7.6 The Anchor Cell as signalling hub

The AC is a very active cell throughout its life-time (Fig.2). It is in fact not born, but selected by LIN-12/Notch signalling from two equally competent cells [29]. The cell producing more LIN-12/Notch ligand adopts the AC fate. It then switches from producing LIN-12/Notch ligand to producing LIN-3/EGF and induces vulval development. After that, the AC instructs uterine development by using LIN-12/Notch signalling again, and at the same time orients vulval symmetry via Wnt signalling. Besides these signals that it is sending, it also receives signals. The most prominent of those is Netrin, guiding it towards the vulva during AC invasion [31]. In the following paragraphs, I will focus firstly on these signalling pathways and secondly on the individual signalling events.

LIN-12/Notch signalling

The LIN-12/Notch signalling pathway is a comparatively simple signalling pathway. Ligands for the LIN-12/Notch receptors are called DSL proteins after the homologues in different organisms: Delta, Serrate (both *Drosophila*) and LAG-2 (*C. elegans*). In *C. elegans*, the DSL family members besides the namesake *lag-2* are *apx-1*, *arg-1*, and *dsl-1* [42]. The LIN-12/Notch receptor is represented by two different proteins in *C. elegans*, LIN-12 and GLP-1. When the receptor is bound by a ligand, the intracellular part of the receptor can be cleaved off by γ-secretase and translocate into the nucleus. Inside the nucleus, the Notch intracellular domain (NICD) activates gene expression as part of a tripartite complex together with LAG-1 and SEL-8 (Fig.4).

It is worth noting that in contrast to most other signalling pathways, it is the receptor itself that translocates to the nucleus to regulate gene expression. No signal amplification via kinase cascades or something similar happens along the way. The relationship between activated receptor and any signalling effect is thus linear. Only when the NICD entered the nucleus

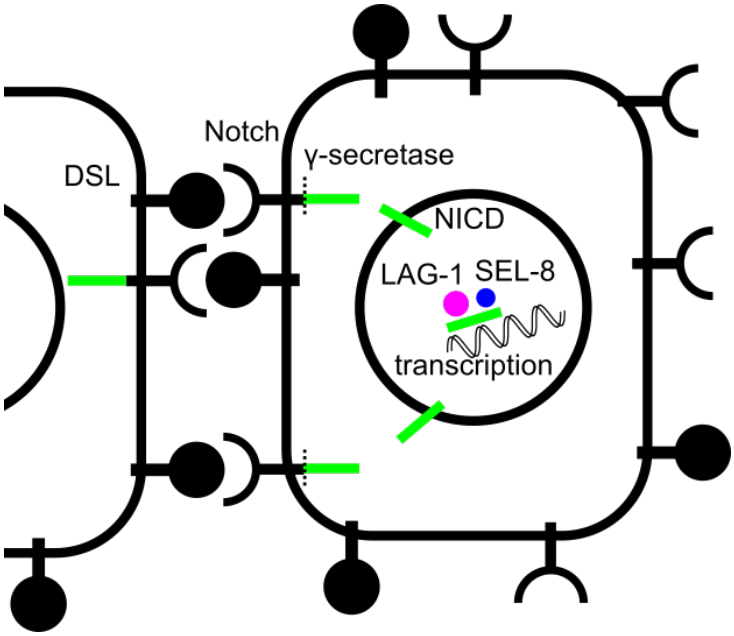

Figure 4: LIN-12/Notch signalling
An illustration of reciprocal LIN-12/Notch signalling, displaying the different steps involved.

can more receptor be synthesised, which forms a positive signalling feedback loop.

LIN-12/Notch signalling is most commonly associated with lateral inhibition and symmetry breaking. It is the linear relationship between activated receptor and transcription that lends itself to this. Because of the linear relationship, even slight, initial differences in ligand concentration lead to a different degree of transcriptional feedback. Most other signalling pathways include amplification of the signal between ligand binding and transcriptional response. This amplification makes the response in different signal receiving cells equally strong regardless of minor differences in active receptor.

Nonetheless, LIN-12/Notch signalling is not only used for symmetry breaking, but can also have the more traditional, instructive effect.

LIN-12/Notch signalling during Anchor Cell specification

At the beginning of the AC's existence stands the classical lateral inhibition. The two cells Z1.ppp and Z4.aaa, descendants of the two somatic gonad founder cells, have the potential to be the AC [43]. In fact, in the absence of LIN-12/Notch signalling, both cells will adopt the default AC fate.

LIN-12/Notch signalling inhibits AC fate [28]. The cell that expresses more LIN-12/Notch ligand, *lag-2*, will inhibit the other cell from becoming AC and stop the lateral inhibitory signal. So in a LIN-12/Notch gain-of-function background, there will be no AC because both potential cells have been inhibited. Besides inhibiting AC fate, the LIN-12/Notch signal suppresses *lag-2* expression and enables the feedback loop needed for lateral inhibition.

For a long time, it has been thought that the decision which cell adopts the AC fate is purely random. However, there seems the be a weak correlation between relative time of birth and becoming AC. The cell that is born first has a higher chance of being the AC. Also, *hlh-2* expression levels correlate positively with acquiring AC fate, which suggests that *hlh-2* upregulates *lag-2* expression [30].

LIN-12/Notch signalling during π cell fate specification

As mentioned, LIN-12/Notch signalling is the lateral inhibitory signal required for AC specification. It also continues during later stages of vulval and uterine development. It is important within the vulval epithelium for 2° vulval fate [28] and then for the specification of a particular uterine cell fate, called the π fate [44]. The uterus is subdivided into dorsal and ventral uterine cells, basically two layers of cells, both consisting of two rows. Within the ventral uterine cells, six adopt the specialized π cell fate that form the connection to the vulva. The AC selects these cells through expression *lag-2*, inducing LIN-12/Notch signalling. Notably, in contrast to AC specification, this event of LIN-12/Notch signalling is not reciprocal or inhibitory, it is instructive. Only the AC expresses the ligand and sends a signal to a subset of the ventral uterine cells. To my knowledge, it is not known whether the AC continuously expresses *lag-2* throughout its lifetime or whether *lag-2* expression is restricted to the developmentally relevant times, such as AC and π cell specification.

LIN-12/Notch signalling – Conclusion

The two instances of LIN-12/Notch signalling described here are very similar on a molecular level, even though the mode of operation is quite different. In one case it is lateral signalling between two equally competent cells, in another it is an instructive signal from an organising cell to select from a subgroup of competent cells. Both the LIN-12/Notch ligand *lag-2* and the receptor *lin-12/Notch* are the same. In fact there is another instructive signalling event within the VPCs that utilizes the same two molecules. It

is the 1° vulval cell instructing its neighbours to become 2°. However, the other molecules involved downstream of Notch are different for each process. This is illustrated by mutants defective in one but not the other processes. For example, *egl-13/cog-2* is needed for π cell specification but not for AC or 2° cell specification [45]. And *dep-1*, even though it is not a Notch target, is part of the pathway that ensures 2° vulval cell fate specification but not AC or π cell [46]. Which factors are involved in AC specification is not clear. The negative feedback loop and its establishment are reasonably well understood. However, which factors specify the default AC fate or the non-default VU cell fate downstream of LIN-12/Notch is unknown. The transcription factor responsible for establishing AC competence and LIN-12/Notch signalling during AC/VU specification, *hlh-2*, is reportedly also required for AC specification [35]. Because it is used repeatedly during AC/VU specification though, the evidence is not very convincing. Nonetheless, whichever factors might be downstream, it is those that distinguish this process from other instances of LIN-12/Notch signalling.

LIN-3/EGF signalling

Epithelial growth factor signalling is an example of the classical receptor tyrosine kinase (RTK) signalling pathway. I will focus on EGF/EGFR signalling because this particular installment is of interest to this thesis. In *C. elegans*, the ligand LIN-3/EGF enables dimerization of the receptor LET-23 and subsequent reciprocal phosphorylation [47]. The signal is then relayed via the SEM-5 adaptor molecule, activating the well known GTPase Ras (LET-60 in *C. elegans*) via its GEF SOS-1. Activated LET-60/Ras in turn activates Raf/LIN-45. The cascade is continued by activation of MEK with the help of KSR scaffold proteins. The last kinase in the chain is ERK/MPK-1 (Fig.5). MPK-1 turns on effectors, for example LIN-1, an Ets transcription factor. This overview is pretty simplistic and fails to highlight all the complex regulatory and branching patterns this signalling cascade has. For example, LET-60/Ras does not only activate Raf, but can have other downstream targets that will enable other downstream effectors. Also, these signalling pathways are not linear, but can and do branch off and interact and integrate into others, and other signalling pathways modulate signalling of this pathway. Regulation and activation of the pathway is complex and can be modified by various co-factors that might be present only in specific cell types.

Nevertheless, there are a few concepts that I want to highlight. First, small GTPases such as LET-60/Ras are an integral part in many signalling

pathways. Basically, they are proteins that have GTP hydrolase activity. In the active form, they are GTP bound and activate downstream signalling components. In the case of LET-60, LIN-45 gets activated. In the GDP bound state they are inactive and do not signal. There are regulatory proteins that control whether these GTPases are GTP bound (active) or GDP bound (inactive). The regulators are called GEFs and GAPs. GEFs, guanine nucleotide exchange factors, stimulate replacing GDP with GTP, activating the GTPase. In the above mentioned LIN-3/EGF signalling, this role is taken by SOS-1 [48]. GAPs, GTPase activating proteins, stimulate the activity of the small GTPase, resulting in the hydrolysis of the bound GTP into GDP, rendering the GTPase inactive. It is primarily GAP-1 that does this in the case of LET-60 [25]. Second, the signal travels via a cascade of kinases until it finally modulates gene expression. This cascade serves two main purposes: Amplification and regulation. Every kinase molecule activated can itself activate several molecules of the next downstream kinase. This amplifies the strength of the signal measured by activated kinases exponentially. Secondly, whenever a kinase is activated, a complex interaction of various proteins happens. At this point, many different signals can be integrated into the pathway and fine-tune the strength of the transmitted signal.

Compared to LIN-12/Notch signalling, this pathway is complex in regulation and components. This makes it flexible and able to respond to several different inputs. Due to the nature of its components, the signal can be amplified or dampened on its way from the cell surface to the eventual transcriptional effectors, making a more graded response possible.

UNC-6/Netrin signalling

Both LIN-12/Notch and LIN-3/EGF signalling, and RTK signalling in general, primarily affect gene expression and cell fate. Unc-6/Netrin signalling primarily affects cell polarity and the cytoskeleton, but there exist reports that also Netrin signalling can be instructive [49].

UNC-6/Netrin signalling is mostly known for its guiding role in axonal migration [40, 50]. The ligand UNC-6/Netrin can be both attractive and repulsive based on the receptors. The main UNC-6/Netrin receptor is UNC-40/DCC, and I will be focussing on its role. Signalling via UNC-40 leads to attraction, the co-receptor UNC-5 reverts attraction to repulsion. Briefly, activated UNC-40 can recruit the TRIO protein UNC-73. UNC-73 is a GEF for the small GTPase Rac, present as CED-10 and MIG-2 in *C. elegans*. Rac can polymerize actin and stimulate the formation of filopodia. In parallel

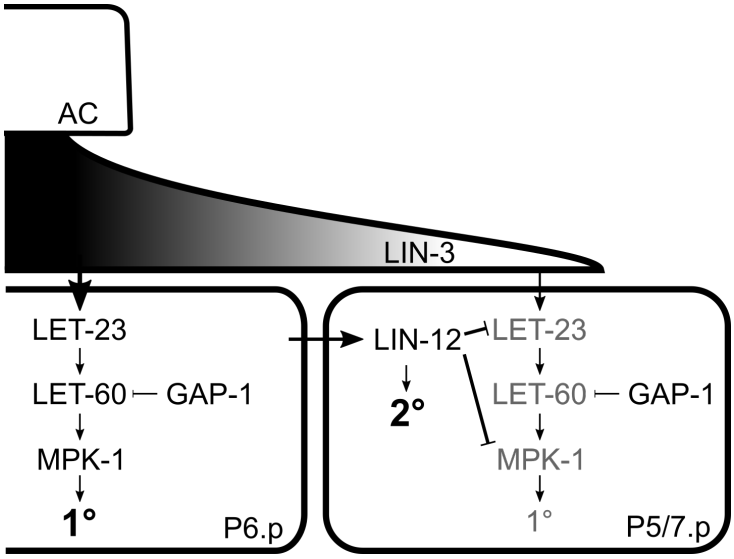

Figure 5: RTK signalling during vulval induction
Inductive signalling in the VPCs. Shown are the major component of the signalling cascade and *gap-1* in the 1° and 2° cell types. Grey indicates lessened activity.

to Rac, UNC-40 can also stimulate actin polymerization via the Ena/VASP homologue UNC-34 [51].

Actin dynamics and its regulation are complicated. Briefly, there are two main regulators of actin and they have been traditionally viewed as antagonists, the small GTPases Rho and Rac [52]. Rac is better known for polymerizing actin into thinner bundles. Rho activates myosin, the actin motor protein, via Rho kinase. This leads to a retrograde actin flow and the formation of actin sheets. However, newer research suggests that especially in vivo both Rho and Rac are required in conjunction for any kind of actin based force generation. It seems to be the balance of Rac versus Rho and where they are active relative to each other that determines which kind of actin dynamic can take place [53]. Presumably the prevailing textbook notion of Rho versus Rac derives from observations done in the overly reductionist environment of Petri dishes where cell can generate force with only one type of GTPase. *In vivo* however, the demands are more complex and so is the required regulatory response.

UNC-6/Netrin signalling during Anchor Cell invasion

The process of AC invasion itself is discussed in more detail elsewhere (see

5.7.4). Briefly, it requires the AC to cross the borders of its original tissue to form a connection between uterus and vulva. This process of crossing tissue borders rarely occurs under healthy, developmental conditions.

Guidance is key during invasion. Two signals provide guidance, but only one is known. UNC-6/Netrin is the known signal [31]. The Netrin ligand *unc-6* in *C. elegans* is reportedly expressed from the ventral nerve cord and possibly from the 1° vulval cells [41]. Presumably, UNC-6/Netrin diffuses from its originating cells at random and forms a concentration gradient. It is sensed by its receptor UNC-40 expressed in the AC. In the AC, activated UNC-40 stimulates actin polymerization and thereby cell migration and invasion.

5.7.7 The Anchor Cell as model

The AC of *C. elegans* is a model for cell invasion since relatively recently only. However, it quickly became widely known even beyond the field of *C. elegans* research. What makes the model special is the ability to observe and manipulate it *in vivo*. This sets it apart from most other models used to study cell invasion that usually either involve collagen coated Petri dishes or chicken allantoic membrane. In these models, not just the substrate for invasion is artificial, but the cells under study are too. In most cases, human cancer cell lines are used as model. These cell lines are immortalized and selected for their ability to cross various barriers put before them, for example collagen. Since relatively recently only, these studies start taking into account not just the invading cells, but also the tumor associated cells. This is a step towards closer replication of the *in vivo* situation. However, using cell lines and artificial substrates for invasion assays makes it difficult to conclude anything specific about either tumor metastasis or cell invasion. By comparison, the AC offers the possibility to study a physiological process *in vivo*.

AC invasion is regulated and influenced by several different mechanisms. The ability to invade is regulated mainly by the transcription factor *fos-1* [38] and the AC is guided by two different signals [31]. Basal laminae breaching itself is complex and requires various cell-cell and cell-basal lamina interactions [54]. At the same time, the AC is surrounded by other cells, sending and receiving various signals to coordinate development of the uterus and the vulva.

AC invasion alone consists of many different steps and processes. In addition to invading though, the AC also regulates and organises the development of uterus and vulva. Treating the AC as a model for invasion alone neglects many of its roles and might ignore important aspects of its function.

5.7.8 How the Anchor Cell is studied

To study the AC, a lot of markers have been developed by the Sherwood lab, focussing especially on the process of invasion. Using AC specific promoters combined with fluorescently tagged proteins of interest, it is possible to highlight specifically the AC and even different selected properties, like polarity, of the AC alone. Targeted and specific manipulations, other than microscopy based ones like cell ablation, are difficult to achieve in the AC. Even though cell-specific enhancers exist, achieving precise control is difficult due to the quickly changing role of the AC during development. Also, the genetic toolkit in *C. elegans* is lacking compared to *Drosophila*. Every tool has to be made individually, no two-factor system like Gal4-*lacZ* offering reusability and easy combination is as established in *C. elegans* as it is in *Drosophila*.

Doing biochemical or high-throughput genomic experiments, i.e. transcriptional profiling, is difficult too, because the AC is only one cell. Experiments of these kinds require a high amount of material, DNA, membrane, proteins, etc., and one cell does not offer the required quantities. A way to work around these restrictions would be to isolate the AC from many animals, even though this provides new challenges.

Another slight hindrance in the study of the AC is that its lifetime stretches across three larval stages, separated by two molting events. These molting events require movement to complete. Any methods relying on prolonged observation and immobilization are thus very likely to lead to developmental abnormalities or death when stretching across more than one larval stage.

In conclusion, the toolset to study the AC is rather limited. This might cause concern when trying to understand the more intricate details of the different roles the AC plays during development.

5.7.9 What makes the Anchor Cell special

What popularized the AC as a model is that it offers the possibility to study a cancer metastasis related process, AC invasion, in an *in vivo* environment. And invasion is not just related to metastasis, but also conserved on the molecular level. Besides AC invasion, the AC features in other important developmental processes, which makes it a very interesting and versatile model. But the many different roles might also complicates drawing relevant conclusions. The AC plays a highly specialized role during *C. elegans* uterine and vulval development. Whether comparable cells exist in humans or other organisms is thus questionable. The complexity of the AC on the one hand

can be considered a benefit, because it allows different processes to be studied, on the other hand it can also muddle potential conclusions and make it more difficult to extrapolate to other systems.

5.7.10 The Anchor Cell as a model for human development

As mentioned, the invasion process of the AC is related to the behaviour of metastatic tumor cell lines *in vitro*. EMT, or epithelial-mesenchymal transition, describes how cells can acquire invasive capabilities in either development or disease and aspects of it seem be common to AC invasion and metastasis. EMT is characterised by epithelial cells losing attachment to their neighbours and apical-basal polarity and starting to migrate and invade [55, 56]. Metastasising tumor cells undergo the classical type 3 EMT that enables them to break away from the primary tumor and enter the bloodstream through invasion to colonize other tissues. During invasion, the AC shows signs of EMT, or more precisely the acquisition of migratory and invasive mesenchymal characteristics. Also the transcription factors involved in EMT and AC invasion overlap to a certain degree. It keeps its tight connections to its neighbouring cells and its polarity intact, however. What is not clear is how much the partial EMT of the AC corresponds to the typical EMT of tumor cells and how similar AC invasion and tumor cell metastasis *in vivo* are. Also, a good point can be made that it is difficult to relate the AC to other cells, because of its multiple, changing roles, and its two apical sides. Additionally, the composition of the extracellular basal laminae that form the tissue borders in *C. elegans* are different from human. Nevertheless, the conservation on the molecular level clearly connects the AC invasion process to metastasis of human cancers. In the future, the interest will be to see whether the AC might also serve as model for the others roles it plays, be it as inductive center and growth factor secretor or coordinator of organ development. These roles are not studied so far for a reason, they are not models for any major human disease. Despite that, the question whether equivalent organizing roles exist in humans or whether it is the limited cell number of *C. elegans* that forces one cell to adopt so many diverse roles is interesting. Maybe cells with even more diverse roles exist in humans because of the increased complexity. However, the kind of investigations that can answer these questions require long-term *in vivo* observations and are difficult to pursue in humans. Finding a similar organizing center in humans would not be totally unexpected, because the need to coordinate the development of different organs will only increase with the complexity of an organism, and the AC might very well help to understand the complexity and requirements of filling many different roles. But no-one yet imagines the

understanding of the AC in its full complexity and to use it not just as a model for its individual tasks, but as model for an organizer and coordinator of development.

5.8 The Vulva

The vulva is the egg-laying organ of *C. elegans*. The vulva also enables male mating for sexual reproduction. During development, the vulva is formed by three cells [29]. These cells go through three rounds of cell divisions producing the 22 cells of the adult vulva (Fig.6). A competence group of six cells have the potential to develop into vulval cells. These cells are called the vulval precursor cells (VPCs). All six cells are equally competent to adopt a vulval cell fate – with exception of P3.p which is slightly less competent – but only P5.p, P6.p, and P7.p become vulval cells in the wild-type. P5.p and P7.p adopt the 2° vulval cell fate and P6.p the 1°. The 2° cells will have seven descendants each. The 1° cell, after three complete rounds of cell divisions, has eight descendants. These 22 adult vulval cells each have one of seven specific sub-fates and are arranged mirror-symmetrically. During vulval morphogenesis, they find the cells with the same sub-fate to form seven toroids around the vulval midline. Five of the toroids fuse and two stay as individual cells [57]. The most dorsal vulval toroids connect the vulva to the somatic gonad to ensure passage of the eggs and embryos.

5.8.1 Vulval development

Vulval development is a paradigm for how many genetic pathways function, especially EGF/RTK, Notch, Wnt, and Netrin, as these are directly involved. As partially mentioned before, the AC is the initiator of vulval development and intimately involved. The AC is the source of LIN-3/EGF that provides the signal that selects the 1° vulval cell from a group of six VPCs. Competence is established through a combination of Hox genes (*lin-39* and *mab-5*) and a Wnt signal [29]. The LET-23/RTK LET-60/Ras pathway activated by LIN-3/EGF triggers the 1° cell specific expression of an inhibitory signal preventing other cells from activating the same LET-60/Ras pathway. Since this inhibition is relayed via LIN-12/Notch, it is short ranged and affects the neighboring cells only. In a wild-type situation, the neighboring cells are P5.p and P7.p, the principal cell receiving the strongest LIN-3/EGF dosage is P6.p. Arguments have been made that an intermediate dose of LIN-3/EGF can trigger 2° fate, which is supported by an alternative LET-60/Ras pathway member, *ral-1*, involved in 2° fate specification [58, 59]. It is not clear how important this LIN-3/EGF dependent signal is for 2° fate acquisition

relative to the importance of LIN-12/Notch. LIN-12/Notch clearly is the dominant player, because strong LIN-12 loss-of-function eliminates 2° cells and ectopic 2° cells form due to dominant LIN-12/Notch alleles [28].

Once cell fates are determined at the beginning of the third larval stage, around the time of the first division of the VPCs, differences start to be visible between 1° and 2° cells. Possibly the first difference on a phenotypic basis is the 1° cell descendants completing the second round of cell divisions before the 2° cells. After all vulval cells have completed the second round of divisions and the vulva is in the so-called four cell stage, morphogenesis of the vulval epithelium starts. It start by transitioning from a one dimensional file of cells into a two dimensional arrangement when the 1° cells push dorsally. Shortly before morphogenesis starts still, the AC breaches the tissue border between uterus and vulva. It is not clear whether this breaching is required for morphogenesis and whether the AC is involved.

After morphogenesis started, a final round of cell divisions follows and creates the 22 cells that constitute the adult vulva. Following that, the two-dimensional cell arrangements turns three-dimensional and the seven vulval toroids start stacking up. Also, the vulval-uterine connection will be formed towards the end of the fourth larval stage and the AC will fuse with a cellular syncytium called utse, freeing up the passage between uterus and vulva. Finally, towards the very end of the larval stages, the vulva everts by collapsing its lumen and thus acquiring the adult structure.

5.8.2 Vulval induction

As mentioned, six vulval precursor cell make up the vulval competence group. Either LIN-3/EGF or LIN-12/Notch is sufficient to induce the vulval cell fate. Depending on the type of signal, the cells either become 1° (LIN-3) or 2° (LIN-12) vulval cells. The AC is the source of LIN-3/EGF under wild-type conditions. It is postulated that the AC secretes LIN-3/EGF in a gradient fashion based on indirect evidence [58]. The presumptive 1° cell is the source of the second signal LIN-12/Notch. The cell receiving LIN-3/EGF turns on production of the LIN-12/Notch ligand LAG-2. This signals the neighbouring cells to ignore the LIN-3/EGF signal and adopt the 2° vulval cell fate.

LIN-3/EGF is sensed by the homologue of the EGFR, LET-23. LET-23 signals via adaptors to LET-60, the Ras homologue of *C. elegans*, which indirectly activates *mpk-1* and the executors of the 1° cell fate. As always, there are many regulatory components of the pathway. I want to point out *gap-1*, which weakly inhibits LET-60 [47, 25].

LIN-12/Notch signalling in the 2° cells is more direct in the activation of its effector genes. But also LIN-12/Notch signalling branches, notably to

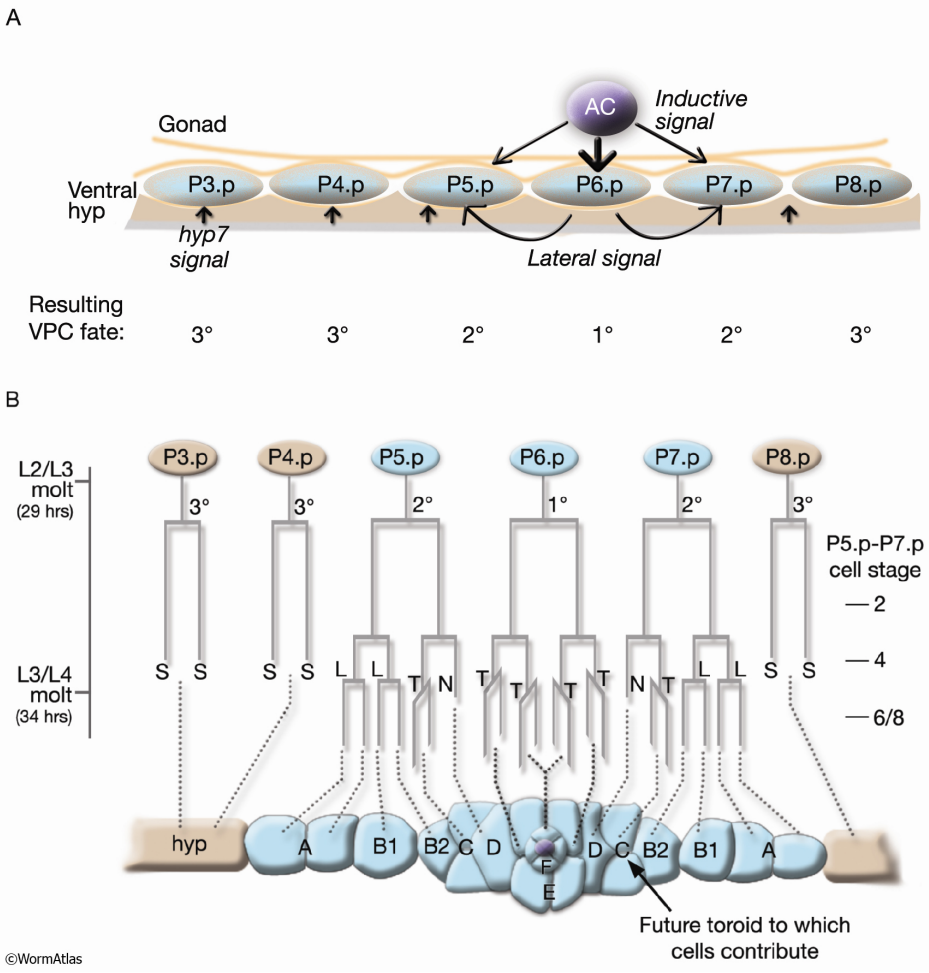

Figure 6: Vulval development
Development of the vulva from (A) induction through to (B) the end cell divisions and the start of morphogenesis. Developmental timings are shown on the left and the stage of cell division of the 2° cells on the right. Illustration taken from wormbase.org

downregulate LIN-3/LET-23 signalling via DEP-1 and LIP-1 [60, 46].

Not just the AC, but also the hypodermis can produce LIN-3/EGF. The hypodermis does so only in mutant situations. Two classes of SynMuv genes repress LIN-3/EGF expression in the hypodermis. When genes of both classes are mutated, LIN-3/EGF is no longer repressed and the hypodermis starts producing LIN-3/EGF, which increases the number of vulval cells greatly.

5.8.3 Vulval induction and LIN-3/EGF signalling from the Anchor Cell

At the beginning of vulval development, three of six potential cells are selected to form the adult vulva. There are two signals involved in this process and there are two different models for how this process works. The two signals are LIN-3/EGF and LIN-12/Notch, the two models are a morphogenetic model in which the LIN-3/EGF signal specifies all cell fates and a sequential signalling model which also includes LIN-12/Notch signalling (Fig.5)[58, 61]. While final proof for the validity of morphogen based vulval induction is missing, I will nevertheless focus on the LIN-3/EGF signal, because this signal originates from the AC and the LIN-12/Notch signal is produced within the vulval (precursor) cells themselves.

lin-3 expression in the AC is regulated differently from other instances of *lin-3* expression. A small deletion has been identified that abolishes AC-specific *lin-3* expression only [62]. It has been suggested that *hlh-2*, the transcription factor involved in AC specification, and unknown nuclear hormone receptors are responsible for expressing *lin-3* in the AC. The expression levels of *lin-3* during vulval induction are extremely high, but also tightly controlled. If *lin-3* RNA expression levels change only two-fold, vulval induction is compromised [63]. After LIN-3/EGF production, how it is exactly transported and how it reaches the vulval cells is unclear and is the focus part two of this thesis. It is postulated that, just like any morphogen, LIN-3/EGF forms a gradient [29]. Supporting evidence for this comes from the finding that the LIN-3/EGF receptor LET-23 can reduce vulval induction [25]. The model is that it does so by sequestering LIN-3/EGF and thereby limiting its diffusion. Expression of LET-23 in P6.p alone is sufficient to stop LIN-3/EGF from diffusing. The ability of LIN-3/EGF to diffuse in turn would support the formation of a growth factor gradient. What is quite clear however, is that P6.p receives the highest LIN-3/EGF dosage in a wild-type condition. This triggers high levels of LET-23/RTK and LET-60/Ras activity in P6.p which finally leads to expression of genes defining the 1° vulval cell fate and at the same time activating the lateral inhibitory LIN-12/Notch

pathway.

5.8.4 The Vulva as model

The *C. elegans* vulva is a very powerful genetic model for several reasons. Genetics, doing crosses, is easy in *C. elegans*, the phenotypes are readily observable and quantifiable, and the vulva is dispensable for viability. If possible, quantification of phenotypes is always desirable. In the vulva, this is particularly easy. The vulva develops from a competence group of six cells. In a wild-type situation, three of those acquire a vulval cell fate, which is visually distinct from the non-vulval one. Every cell that is induced can be identified individually. And since in a wild-type situation, only half the cells are induced means that mutants can skew the number of induced cells both ways.

Furthermore, no less than three different signalling pathways cooperate to induce vulval development. The EGF/RTK, Notch, and Wnt pathways all play a major role. More recently, the vulva also became a focus of morphogenetic studies as a model for epithelial tube formation. Again, observation and microscopy is easy and also enables laser manipulation for targeted cell killing or cutting of subcellular structures like actin bundles.

Both signalling and epithelial tube morphogenesis are central to development and because the vulva is so easy to observe and manipulate, both physically and genetically, it has been a popular and successful model for many years. One could argue that vulval development is fully understood and as such no longer a useful model to study. Indeed, the interactions of the major pathways and their components have been for the most part deciphered. But there are always details that are not understood or processes that have been overlooked. If anything, I would argue that the problem with studying the *C. elegans* vulva is not that it is fully understood, but that we think everything is understood. There are still many aspects, e.g. morphogenesis, AC invasion, and even induction, that are only partially understood, and the vulva will continue to be a great model to study different aspects of development for the foreseeable future.

5.8.5 How the Vulva is studied

In contrast to the study of the AC, the focus of vulval development is not primarily the subcellular distribution of various proteins or lipids, but the acquisition of the various cell fates. A comprehensive set of marker genes identifies all seven different specialized vulval cell fates and allows cell-type specific gene expression [64]. However, for basic experiments concerned with

vulval induction alone, markers are not required. As mentioned above, the vulva is easily manipulated with either genetic, physical, or chemical methods, and typically, manipulations do not impact on development in general.

For studying morphogenesis, the toolset is not as well established as it is for cell fate specification. But a basic set of markers for cytoskeletal, junctional and membrane-bound proteins is available. Vulval morphogenesis stretches over two larval stages. Because of this, observing the whole process *in vivo* is difficult due to molting. However, the formation of the vulval tube can be observed as a whole.

High-throughput analyses are subject to similar restrictions as when studying the AC. The vulva is a small part of the whole worm, and even though markers are available, isolation would be difficult. Furthermore, the vulva is not a homogeneous tissue, but instead composed of seven different cell-types. Consequently, gathering the necessary material for either transcriptomics or similar experiments is difficult.

5.8.6 What makes the Vulva special

As opposed to the AC as a rather unique model for cell invasion *in vivo*, the vulva is just one of many models for cell fate specification or even tube morphogenesis. However, the vulva stands out in a few aspects. The number of vulval cells is limited, which allows the levels of signalling to be quantified. Almost every component of the pathways involved in vulval induction can be manipulated genetically. This makes studying the individual pathways and their interactions more precise. The vulva is non-essential, and even if worms lacking a vulva have a reduced number of progeny and cannot mate, they nevertheless procreate. This greatly simplifies the study and identification of vulvaless animals. Also even the most extreme cases of vulval induction are not disturbing development. This means that vulval development does not impact on overall development, and consequently, there is no feedback form overall impaired development affecting the vulva. Furthermore, observation is easy and highly informative. Lastly, vulval development is very well studied and the available in-depth studies provide a well-defined system and many readily available tools.

5.8.7 The Vulva as a model for human development

Obviously, cell fate specification is a key process in the development of any system, and the pathways important for for vulval development are conserved from worm to human. Also epithelial tube morphogenesis is central for many different physiological necessities. In humans, epithelial tubes are basically

omnipresent, e.g. as blood vessels or renal tubules. In this respect, the vulva as model is very well suited to study processes important for us humans. However, the same restrictions I mentioned when talking about the AC apply. Mainly, the differences between worm and human lie in the complexity of the system. This does not just refer to the obvious difference in size and cell number, but also in the complexity of the signalling pathways and their components. Most unique genes in *C. elegans* have more than one homologue in humans, and each homologue has its specialized niche and is regulated differently. As a consequence, even though studying *C. elegans* provides a general understanding of how a specific pathway functions, it is not suited to answer specific questions referring to regulation and/or activity.

Studying the *C. elegans* vulva is very well suited to determine a blueprint for how cell fates are specified or what is necessary for epithelial tube formation. However, the blueprint is a very generic and is not suited to establish how pathways are regulated and function in detail in higher organisms, including humans.

5.9 The relationship between Anchor Cell and Vulva

In the course of my doctoral studies, the interaction between the AC and the vulva was central. It is an interaction that might seem unidirectional from the AC towards the vulva at first, but it is in fact a continuing dialogue.

Textbooks say the AC secretes LIN-3/EGF to start vulval development. The next step is AC invasion, guided by the vulva, during which the AC forms the uterine-vulval connections.

It is more complex, and the interactions between AC and vulva are crucial and manifold. For example, we found that AC polarization is not only important for AC invasion, but also during vulval induction, which is the topic of the second part of this thesis. This finding suggests that the VPCs signal to the AC even before vulval induction.

5.9.1 Early interactions

From textbook knowledge it is clear that the AC sends the inductive LIN-3/EGF signal to the vulva to start vulval development. In the second part of my doctoral thesis, we find vulval induction to be more complex. We suggest that the inductive gradient is not the default and needs to be established to guarantee controlled induction. Likely, the VPCs contribute to how the gradient of LIN-3/EGF is formed.

5.9.2 Interactions during vulval induction

During vulval induction, vulva and AC are separated by two layers of basal lamina. Secreted LIN-3/EGF can penetrate the basal laminae and induce vulval development in the VPCs. However, *rom-1*, the homologue of the protease responsible for cleaving EGF in both *Drosophila* and humans, is not required for vulval induction [65, 66, 67]. It is thus not clear how and if LIN-3/EGF is cleaved and whether LIN-3/EGF needs to be secreted from the AC at all. Many observations show vulval induction from a distance, most strikingly in *dig-1* mutants with dorsally located uteri, strongly suggesting that LIN-3/EGF is secreted [68]. Despite that, the mechanism is not known.

5.9.3 Late interactions

Towards the end of the third and during the fourth larval stage, the AC initiates formation of the uterine-vulval connection. First, the AC invades and aids in vulval morphogenesis. Next, the AC specifies the uterine π fate. These π cells will eventually become uv1 cells and link the uterus to the vulva. To do so, they need a vulval signal. LIN-3/EGF is the vulval signal and it is dependent on the transcription factor *egl-38* [69, 33]. Whether the vulva has a role other than inducing uv1 cells is not known.

5.10 Uterine development

After embryonic development is finished and the larva hatched, the germline primordium has four cells. Z1 and Z4 are the precursors of the somatic gonad, including the uterus, Z2 and Z3 are the founders of the germline itself [70, 43]. The cells of the germline primordium start proliferating in the middle of the first larval stage, the germline precursors Z2 and Z3 seemingly uncoordinatedly, and Z1 and Z4 in a more regulated manner. At the end of the first/beginning of the second larval stage, Z1 and Z4 divided thrice giving rise to twelve cells. Two of these cells, Z1.aa and Z4.pp, are the distal tip cells that will lead the migration of the gonad arms and define the stem-cell niche. Another two are the AC precursors Z1.ppp and Z4.aaa. As described previously, these two cells then engage in reciprocal LIN-12/Notch signalling to determine which one of the two will adopt the AC fate. The cell not becoming AC will be one of three ventral uterine (VU) cells. As their counterparts, there are two dorsal uterine (DU) cells. It is these five cells that will form the uterus. The adult uterus is composed of three parts, two distal lobes, each made of four toroids, ut1-4 [71, 32]. The central part of the uterus joins the two lobes and provides the connection to the vulva.

It is this part that is formed by the descendants of the π cells. π cells, as mentioned, are a specialized subset of VU cells, specified by the AC through LIN-12/Notch signalling (Fig.2). The π cells divide and form two distinct cell types, uv1 and utse. The utse anchors the vulval-uterine connection to the lateral seam and is the syncytium that the AC will fuse with, leaving only a thin hymen separating vulva and uterus. The uv1 cells are specified by a LIN-3/EGF signal, not from the AC but the most proximal vulval cells, the vulF cells [69]. The transcription factor *egl-38* stimulates *lin-3* production in the vulF cells. Secreted LIN-3/EGF then specifies the four uv1 cells. These uv1 cells are important for forming the connection between the dorsal vulva and the ventral uterus. If these cells are missing, the vulval-uterine connection remains incomplete and eggs cannot be laid. The uv2 and uv3 cells seal the dorsal part of the central uterus.

Notably, the AC specifies both the central vulval cells as well as the central uterine cells. It serves as the linchpin or anchor that marks where vulva and uterus are centred and where the vital connection between the egg-producing and egg-laying organs is formed.

6 Research questions of this thesis

Two different projects make up this thesis. Both focus on the AC and its polarity, but on two different roles it has. In the first part, I study the invasion of the AC, and ask how *madd-2*, the homologue of the Opitz syndrome gene *Mid1*, is involved. The second part of this thesis is about vulval induction by the AC. The question is whether and how the polarity of the AC affects distribution of the vulval inducing growth factor LIN-3/EGF, and how this influences vulval induction.

Personally, the first part of this thesis is more or less a standard genetic research project. Its question is clearly defined and limited to one gene, *madd-2*, and one process, AC invasion. Somewhat disappointingly, in the 2.5 years since its publication, it has received relatively little attention. Disappointing mostly because I think that the mechanism we described is truly interesting and adds potentially another layer to the regulation of cell invasion. Admittedly, we lack any detailed mechanism, but overall, the phenotype is clearly described. Anyway, the detailed discussion and the publication follow below.

The second part for me is more exciting, simply because it is based on a new way of looking at an old problem. This is fascinating regardless of the topic I think. Essentially, it is the combination of insight from studies on AC invasion into various characteristics of the anchor cell and an old finding that describes LET-23, the receptor for the inductive signal LIN-3/EGF, as

a negative regulator of vulval induction. I hope and think that what we found and describe will highlight an aspect of cell-cell communication and signalling in a new context. This project is not yet finished as I write this thesis, but an incomplete version of the manuscript is included.

7 Part 1 – The *Caenorhabditis elegans* homologue of the Opitz syndrome gene, *madd-2/Mid1*, regulates anchor cell invasion during vulval development

7.1 Introduction

7.1.1 Cell invasion

The process of cell invasion is one that is mostly associated with malignant metastasis. However, it is in fact a crucial component of normal development of multicellular organisms. In *C. elegans*, we know that cell invasion is happening during AC invasion [72], however, it might very likely also be involved in the formation of the excretory system, the duct cell, and during morphogenesis of the pharynx and gut. In mammals, we have a bit broader knowledge. Here, cell invasion is a part of the early stages of pregnancy, formation of the mammary glands and the renal system. So despite its notoriety, cell invasion is an essential process of the formation of functional multicellular organisms. As such, treating *C. elegans* AC invasion as a model for metastatic cancer cells only and focussing purely on the basal laminae breaching aspect of the process is a bit short-sighted. Because the genetic toolkit is comprehensive and microscopy very well established, which makes the study of AC invasion great in the first place, further aspects of cell invasion, such as coordination and interaction between the two tissues, could be very easily studied also.

7.1.2 Cancer

We all know about cancer. It is possibly the most well-known disease of these days. Evolutionary, this is most likely because we never lived longer than today, and because our medical knowledge and detection methods were never as refined.

One way to look at cancer is to see it as an evolutionary step back, to where every cell is trying to survive, instead of functioning as an organism and working towards a set of related cells, the germ cells, surviving.

Cancerogenesis is a complex process, because there are many different roads to cancer, and no two cancers are alike, even though they might share some features. So it is not too surprising that there are so many publications describing every imaginable gene to be involved in some kind of cancer. In the course of cancerogenesis, the cells undergo so many changes, also in term of genome stability, that in a big enough sample, any given gene will be deviating from wild-type. On a more conceptual level, there are several steps in cancerogenesis, like independence of growth factors, loss of contact inhibition, angiogenesis, and invasion or metastasis [73]. Successful metastasis itself is a very complex process and requires the cells to leave their place of origin, cross the barrier into the bloodstream, then cross it again, manage to settle into a new environment and start growing. There are a lot of obstacles and difficulties that need many adaptations before a cell can do this. And the other steps in cancerogenesis are just as complex. This is why usually cancer takes many years to develop. There are different theories as to how cancer cell evolution happens, involving stem cells, gradual acquisition of new traits, etc. Overall, it is not fully understood how cancer develops and what the crucial stages are. In addition, many of the models that are used to study cancer are in fact cell lines derived from cancers. This provides its own set of problems because once cancer cells reached a stage in which they can be kept *in vitro*, they need to have acquired so many new characteristics and can change so rapidly that it is very difficult to draw generally valid conclusions. It is no doubt difficult to study cancer thus, because it is such an inhomogeneous disease. One approach thus that could provide an understanding of cancerogenesis is to try and understand the characteristics of cancer cells such as independence of growth signals, angiogenesis and cell invasion. This approach has the advantage that all these processes individually can be studied in a 'clean', wild-type context. Like that it is possibly to understand better how the process is regulated and how it is executed. this is also what is being done in the case of studying AC invasion in *C. elegans* in an effort to try and understand cancer cell metastasis. What then usually is done to verify any results found in this way is to try and see whether the genes are also important in cancer cells. For the conservation between AC invasion in *C. elegans* and invasion of cancer cells, there is conservation, but it is not great [21]. However, we should start asking ourselves whether this is in fact a meaningful comparison, as one situation presents the process happening in the 'clean', wild-type situation, and the other a highly corrupted, disorganized, heavily modified one. So maybe it should not come as too big a surprise that the conservation is indeed quite low whenever we compare any given process in its wild-type situation to its cancerous counterpart, not because they are in fact not similar or even the same, but because many

other changes happen in the cancerous background that are not relevant to the process itself.

7.1.3 *madd-2*

madd-2, or *Mid1* in vertebrates, is a gene that has many roles across many organisms, and generally, the roles are not conserved. In *Xenopus*, the claw frog, *madd-2* regulates neural tube closure [74]. In chicken, it is involved in regulating gene expression in the Hensen's node during gastrulation [75]. In both *Drosophila* and *C. elegans*, it is important for neuronal migration [76]. It is characterized to guide muscle arm extension in *C. elegans* [77]. In mice, it is needed for brain development [78], and in humans it is associated with an inheritable disease called Opitz G/BBB syndrome, characterized by midline guidance defects, such as cleft lip and palate, including slight mental retardation [79]. How all these different function come about is not well understood, a consensus seems to be the ability of *madd-2* organize the microtubule network. Also, it has a RING finger domain, that can act as E3 ubiquitin ligase, transferring ubiquitin moieties to various proteins and marking them for destruction by the proteasome [80]. This activity has been demonstrated *in vitro* but whether or not it is important for its role *in vivo* is not clearly established. Similarly, *madd-2* associated with protein phosphatase 2A, whether or not that is significant for its function is unclear. *madd-2* is thus a protein that might be a part of multiple processes, one or many of which are conserved and important in human development, but the mechanisms of how it is working in each and every process and whether there is an underlying core function to it all is not known.

In our lab, we got interested in *madd-2* because it was a hit in an RNAi screen for regulators of LIN-12/Notch signalling. Follow-up experiments seemed to show that *madd-2* is indeed involved in LIN-12/Notch regulated processes during vulval development, namely in cell fate specification of two specialized gonadal cell types, the AC and the π cells, as the number of these cells in *madd-2* mutants varied from wild-type. Also, a third phenotype was associated with the AC, a defect in AC invasion. This phenotype was not directly linked to LIN-12/Notch signalling, but at the time the most interesting one. This is due to the similarities between AC invasion in *C. elegans* and cancer cell metastasis in humans.

Because of its association with cancer development and metastasis in particular, this last phenotype, the AC invasion defect, was of particular interest. Also, at the time we started the project, progress had just been made in understanding one of the main guidance signal important for AC invasion. It was thus natural to see whether and how *madd-2* fit into the

established guidance pathways or whether it was involved in regulating a different aspect of AC invasion. These results can be found in the publication I attach here in full.

Both AC invasion and cancer cell metastasis are similar not just to each other, but to many other developmental processes. Basically, cell invasion describes the process by which connections are formed between two different tissues. As can be imagined, crosstalk between different tissues is important for proper bodily function. And although most crosstalk happens across still existing boundaries by means of diffusible signal cues, this is not always sufficient. This is generally the case when the connection needs to do more than just rely information and for example also transport nutrients or in many cases of epithelial migration and specifically for example during embryo implantation at the beginning of a pregnancy or epithelial branching morphogenesis. Even though it might seem that cell invasion is only a part of cancer cell metastasis and thus unwanted and even lethal, it is as a matter of fact an essential part of development. As mentioned in the beginning, this is an overarching principle of cancer development. Cancer cells do not reinvent any of the processes or characteristics that make them dangerous, but just re-employ them outside of the developmental program they were intended for. We are studying cell invasion in the context of metastasis primarily not because metastasis is a good model for understanding invasion but because cancer, and in particular metastasising cancer, is a leading cause of death. And even though we now study AC invasion, which is an invasive process in a well regulated, developmental context, we always aim to compare and relate it to the aberrant rogue process of cancer cell invasion. The effort to study cancer cell invasion is understandable, but other models than AC invasion might yield a better comprehension of invasion *per se*. And understanding invasion should be the first step towards understanding cancer cell invasion.

In conclusion, it is not the model that should closely mimic cancer cell invasion, but it should be characterized in which way cancer cell invasion is mimicking which model. Another reason that speaks in favour of this approach is the heterogeneity of cancers. None two cancers are completely alike, as a consequence of cancer associated genome instability. Even if there are factors commonly associated with the invasive, metastatic phenotype of cancers that we are trying to understand by studying AC invasion, there will surely be other ways for cancer cells to metastasis. So we should diversify the model we study so we have multiple points of reference to which we can relate cancer cell metastasis.

Despite a lot of interest in and research into AC invasion, a lot is not understood still. Starting at the beginning, hardly any major components that constitute the invasion machinery are known. The genes *zmp-1*, *cdh-3*,

and *him-4* are the ones known to have an apparent direct involvement in dissolving the basal laminae [38]. However, even the triple mutant only results in a delayed invasion in 25% of all animals, and 98% show full AC invasion by the L4 stage, with none having a defect. It is thus sorely obvious that we actually do not understand how invasion happens, even though we have a fairly good model of how it is regulated and guided. Evident by the fact that no major executors of invasion are known is that the actual machinery is composed of many, individually negligible genes. So the standard genetic method of finding the genes responsible for breaching the basal lamina does not work. There might currently be efforts underway to try and do single cell transcriptomics of the AC in order to find genes that are upregulated during invasion. Such an experiment, if successful, might indeed provide insight into the identity fo said invasion executors. Alternatively, using the triple mutant as sensitized background for further screens might work, however, any mutant background, triple mutants especially, tend to start deviating significantly from any wild-type like conditions. An approach that to my knowledge has not been tried in *C. elegans* yet, would be to try and rescue invasion defective mutants by injecting constitutively expressed transgenes of candidates. For example, one might try to reduce the invasion defect arising from a loss of the master regulator *fos-1a* by injecting a heat-shock promoter driven invasion executor. And such an approach could relatively easily be tested, because some executors are known.

Another unknown is the second guidance signal besides *unc-6/Netrin*. It is only known that there is at least one other cue and that this cue is produced in the 1° vulval cells [72, 66, 31]. What the cue is and how it is sensed by the AC is not known. However, it presumably triggers the same downstream pathway in the AC as does *unc-6 unc-40* signalling. It might just be the strength of the signal that is enhanced by the additional cue, because even without *unc-6*, in most cases invasion does not fail, but is simply delayed.

Indeed, what we know about AC invasion is rather bare-bones. The master transcriptional regulator *fos-1*, a downstream transcriptional regulator *egl-43*, some executors *cdh-3*, *zmp-1*, *him-4*, some components of cellular integrity are necessary, namely the integrins, and then the guidance system comprised of *unc-6* and *unc-40* [72, 38, 31, 81, 39]. All else that is required for making the AC invasion competent, potential negative regulators, and the missing pieces of the basic machinery are unknown.

madd-2 turned out to be a potential negative regulator, with possible ties to the *unc-6/unc-40* guidance system.

7.2 Publication

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The *Caenorhabditis elegans* homolog of the Opitz syndrome gene, *madd*-2/*Mid*1, regulates anchor cell invasion during vulval development

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ABSTRACT

Mutations in the human *Mid*1 gene cause Opitz G/BBB syndrome, which is characterized by various midline closure defects. The *Caenorhabditis elegans* homolog of *Mid*1, *madd*-2, positively regulates signaling by the *unc*-40 *Netrin* receptor during the extension of muscle arms to the midline and in axon guidance and branching. During uterine development, a specialized cell called anchor cell (AC) breaches the basal laminae separating the uterus from the epidermis and invades the underlying vulval tissue. AC invasion is guided by an UNC-6 Netrin signal from the ventral nerve cord and an unknown guidance signal from the vulval cells. Using genetic epistasis analysis, we show that *madd*-2 regulates AC invasion downstream of or in parallel with the Netrin signaling pathway. Measurements of AC shape, polarity and dynamics indicate that MADD-2 prevents the formation of ectopic AC protrusions in the absence of guidance signals. We propose that MADD-2 represses the intrinsic invasive capacity of the AC, while the Netrin and vulval guidance cues locally overcome this inhibitory activity of MADD-2 to guide the AC ventrally into the vulval tissue. Therefore, developmental cell invasion depends on a precise balance between pro- and anti-invasive factors.

Introduction

Mutations in the human *Mid*1 gene cause Opitz G/BBB syndrome (OMIM 300000), which is characterized by various midline closure defects ranging from hypertelorism (widely spaced eyes) to cleft lip and palate (Quaderi et al., 1997). MID1 functions as an E3 ubiquitin ligase that targets the protein phosphatase PP2Ac for degradation. Moreover, MID1 binds to and is transported on microtubules and is proposed to regulate protein translation (Aranda-Orgillés et al., 2008a, 2008b; Schweiger et al., 1999; Trockenbacher et al., 2001). The vertebrate homologs of MID1 regulate microtubule stability, asymmetric gene expression and brain development (Granata and Quaderi, 2003; Lancioni et al., 2010; Suzuki et al., 2010). *madd-2,* the sole *Caenorhabditis elegans* homolog of *Mid*1, is required for the extension of muscle arms toward the ventral midline and for axon branching and guidance (Alexander et al., 2010; Hao et al., 2010). In both of these processes, MADD-2 positively regulates signaling by the UNC-40 Netrin receptor.

Cell invasion, the breaching of tissue borders, is crucial during a variety of developmental processes, such as vertebrate kidney development or implantation of the early embryo into the uterus. Invasion is not only required during normal development, but also plays a key role during cancer progression. An epithelial to mesenchymal transition leading to an invasive phenotype of individual cancer cells is the first step during metastasis formation (Kraljevic Pavelic et al., 2011).

In the *C. elegans* larva, a specialized uterine cell called anchor cell (AC) first induces vulval development by secreting the EGF-like growth factor LIN-3 (Sternberg, 2005) and then invades the vulval tissue (Sherwood and Sternberg, 2003). AC invasion begins during the third larval stage after the second round of vulval cell divisions (the Pn.pxx stage). During AC invasion, two basal laminae (BL) between the uterus and vulva must be breached, which involves two distinct mechanisms: Guidance and BL breaching. AC guidance depends on UNC-6 Netrin expressed by cells in the ventral nerve cord (VNC) and an unknown signal expressed by the 1° vulval cells (Fig. 1A) (Ziel et al., 2009). BL breaching, on the other hand, requires the transcription factors FOS-1A and EGL-43L, which induce the expression of several effectors of BL breaching (Rimann and Hajnal, 2007; Sherwood et al., 2005). Some of the genes required for AC invasion possess mammalian homologs that have been implicated in regulating the invasiveness of human cancer cells (Hagedorn et al., 2009;

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Matus et al., 2010; Schindler and Sherwood, 2011). However, so far only genes that positively regulate AC invasion have been identified in *C. elegans*, and it is not known whether AC invasion must also be actively repressed.

Here, we show that MADD-2 regulates AC invasion by repressing the formation of ectopic membrane protrusions. We propose that in order to achieve a directed, precise invasion of the AC into the vulval tissue, undirected cell invasion must first be suppressed by MADD-2 and then overcome by the specific guidance cues from the VNC and vulval cells.

Results

madd-2 is expressed in the invading AC

The *madd*-2 gene (C39F7.2) was identified in an RNAi screen for genes involved in vulval fate specification and morphogenesis (Rimann, 2008). A functional MADD-2::GFP reporter (Alexander et al., 2010) was strongly expressed in the AC and more weakly in the adjacent uterine cells around the time of invasion (Pn.px to Pn.pxx stages, Fig. 1B, C). The MADD-2::GFP signal was slightly polarized toward the basal side of the AC, where the invasive membrane forms. Moreover, animals carrying the *madd*-2(*tr*103) mutation, a putative null allele (Alexander et al., 2010), displayed AC invasion and occasionally also AC specification defects resulting in AC duplication (Fig. 1E, F, 2 out of 30 animals had two ACs). In the following, we focussed on the role of MADD-2 during AC invasion.

MADD-2 is required for efficient AC invasion

In order to observe and quantify AC invasion, we used MitoTracker staining to visualize the BL separating the AC from the vulval cells (Sherwood et al., 2005). All wild-type animals showed complete BL breaching at the Pn.pxx stage, whereas the AC did not breach the BL in approximately 30% of *madd*-2(*tr*103) mutants (Fig. 2A, B, L). A similarly penetrant AC invasion defect was observed with the *madd*-2 deletion allele *ok*2226 (data not shown), and the *madd*-2(*tr*103) invasion defect was rescued by the translational MADD-2::GFP reporter (Fig. 2L). By the Pn.pxxx stage the AC had breached the BL in most *madd*-2 mutants (48 out of 51 cases), indicating a delay in AC invasion. Next, we examined a LAM-1::GFP laminin reporter (Ziel et al., 2009), which allowed us to create 3D reconstructions of the ventral BL. In the wild-type, the AC formed a single circular opening at one defined site (Fig. 2G). However, in *madd*-2(*tr*103) mutants that exhibited signs of basal lamina breaching when viewed laterally, the opening was either irregularly shaped and less defined (Fig. 2H) or the AC created multiple, smaller gaps in the laminae (Fig. 2I). Thus, *madd*-2 is required to focus the invading AC onto a single site on the BL.

madd-2(lf) suppresses the AC invasion defects in unc-6 netrin(lf) and VPC ablated animals

UNC-6 Netrin signaling is necessary to guide the AC ventrally during invasion (Ziel et al., 2009). Hence, the *unc*-6(*ev*400) null mutation (Hedgecock et al., 1990) prevents AC invasion in around 80% of the animals at the Pn.pxx stage (Fig. 2C, L). Since Alexander et al. (2010) and Hao et al. (2010) found that MADD-2 positively regulates the UNC-40 Netrin receptor during muscle arm extension and axon guidance, we tested whether *madd*-2 functions in a similar way during AC invasion. Surprisingly, *madd*-2(*tr*103) partially rescued the BL breaching defect of *unc*-6(*ev*400) mutants, as *madd*-2(*tr*103); *unc*-6(*ev*400) double mutants displayed a similar frequency of invasion defects (around 30%) as *madd*-2(*tr*103) single

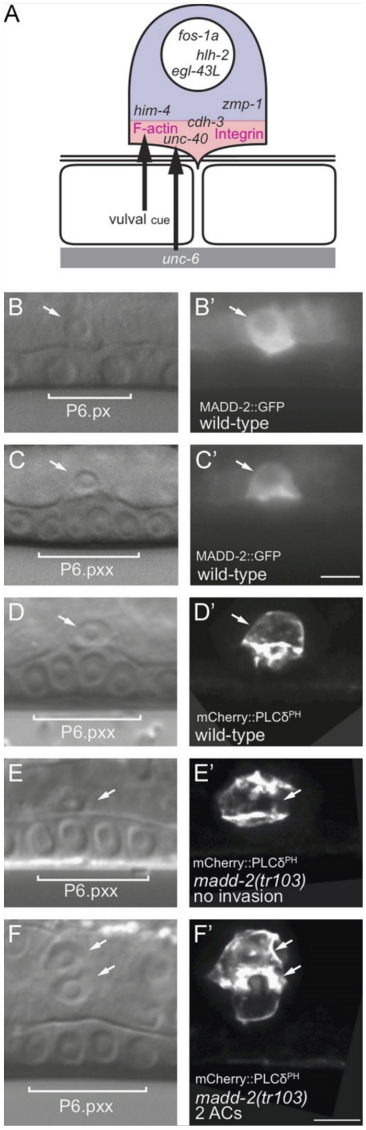

Fig. 1. *madd*-2 is expressed in the invading AC (A) Schematic model of the genes and signals regulating AC invasion. (B) MADD-2::GFP (*tr*Is31) is strongly expressed in the AC and weakly in the surrounding uterine cells at the beginning and (C) during AC invasion. (D) AC invasion in the wild-type, (E) a *madd*-2(*tr*103) mutant with intact BL and (F) a *madd*-2(*tr*103) mutant with two ACs. The ACs are labeled with the mCherry::PLCδ^{PH} reporter *qyl*s23. Arrows indicate the AC and the brackets the 1° vulva cells. Scale bars: 5 μm.

mutants (Fig. 2D, L). Besides the UNC-6 Netrin signal, an unknown cue produced by the vulval cells is also required for ventral AC guidance (Ziel et al., 2009). To investigate the interaction between *madd*-2 and the vulval guidance cue, we ablated the vulval

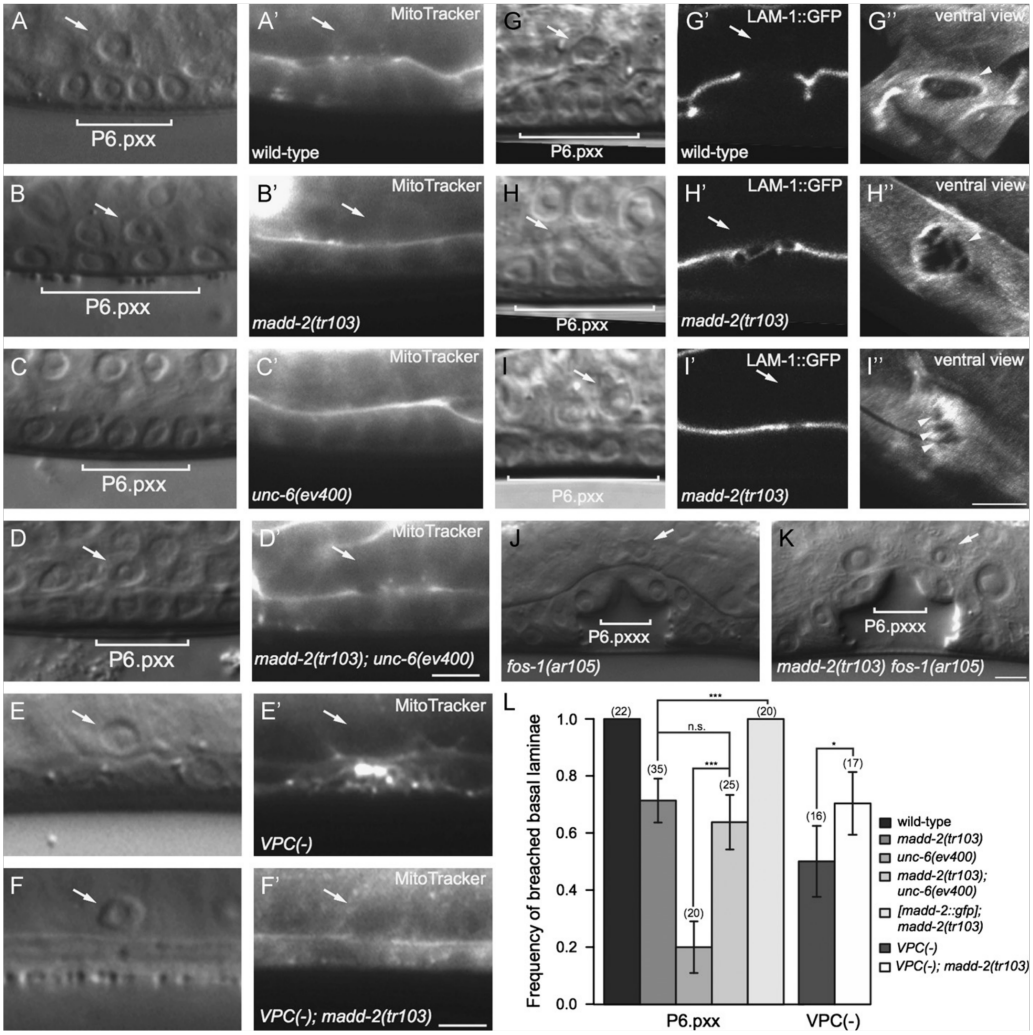

Fig. 2. AC invasion in *madd-2* mutants is *unc-6* and vulva independent but *fos-1a* dependent. (A) Nomarski images of wild-type, (B) *madd-2*(*tr*103), (C) *unc-6*(*ev*400), (D) *madd-2*(*tr*103); *unc-6*(*ev*400), (E) VPC ablated wild-type and (F) VPC ablated *madd-2*(*tr*103) animals at the P6.pxx ((A) through (D)) and mid L3 stage (E,F). In (A′) through (F′) the BL are stained with MitoTracker. (G) Nomarski image, (G′) lateral view and (G″) ventral view of the BL labeled with LAM-1::GFP (*qyIs*10) in a wild-type animal at the P6.pxx stage. (H) through (H″) and (I) through (I″) show two examples of abnormal BL breaching in *madd-2*(*tr*103) mutants. Arrowheads indicate gaps in the BL. (J) Nomarski image of a *fos-1a*(*ar*105) single and (K) a *madd-2*(*tr*103) *fos-1a*(*ar*105) double mutant with intact BL at the Pn.pxxx stage. Arrows indicate the AC and the brackets the 1° vulval cells. Scale bars: 5 μm. (L) Quantification of BL breaching by mitotracker staining at the P6.pxx and mid L3 stages in the genotypes shown above, rescue by the MADD-2::GFP reporter and in VPC ablated animals. Error bars indicate the s.e.m. as estimated by bootstrapping and (*n*) the number of animals analyzed. *p*-values derived from bootstrapping are indicated with $p^{*} < 0.05$, $p^{**} < 0.001$, $p^{***} < 0.0001$ and *n.s.* > 0.05.

precursor cells (VPCs) at the mid L2 stage before vulval induction. Ablation of the VPCs caused an AC invasion defect in around 50% of wild-type animals, while in VPC-ablated *madd-2*(*tr*103) mutants the AC successfully breached the BL in around 70% of the cases, which is similar to the frequency observed in *madd-2* mutants containing vulval cells (Fig. 2E, F, L). We conclude that *madd-2* regulates AC invasion downstream of or in parallel with *unc-6* and the vulval guidance cue.

AC invasion in madd-2(lf) mutants requires fos-1a activity

AC invasion not only depends on guidance signals such as UNC-6 Netrin but also on the activity of *fos-1a*, which gives the AC the capability to remove the BL (Sherwood et al., 2005). We thus tested whether AC invasion in *madd-2*(*tr*103) mutants depends on *fos-1a*. In *fos-1a*(*ar*105) single mutants, the BL remained intact in 34 out of 36 animals (94%) at the Pn.pxxx stage (Fig. 2I). *madd-2*(*tr*103) did

not suppress the invasion defect of *fos*-1*a*(*ar*105) mutants but rather caused a slightly enhancement, as all of 35 *fos*-1*a*(*ar*105); *madd*-2(*tr*103) double mutants examined had an intact BL (Fig. 2J, p-value < 0.0001). Thus, MADD-2 controls AC guidance rather than the invasiveness induced by FOS-1A.

MADD-2 suppresses the formation of ectopic AC protrusions

To gain further insight into *madd*-2 function, we examined AC shape and polarity using the mCherry::PLCδ^{PH} reporter, which binds to phosphatidylinositol-(4,5)-bis-phosphate (PIP_2). Highest PIP_2 levels are observed at the ventral, invasive plasma membrane, where actin polymerizes and AC protrusions are formed (Fig. 3A, B) (Luna and Hitt, 1992; Ziel et al., 2009). In contrast to the bell-shaped appearance of the AC in the wild-type and the round shape in *unc*-6(*ev*400) mutants, the AC in *madd*-2(*tr*103) single and *madd*-2(*tr*103); *unc*-6(*ev*400) double mutants adopted a rectangular shape with irregular borders (Fig. 3A–F, suppl. Fig. s1A). Using the PIP_2 reporter to measure AC polarity, we found that in *madd*-2(*tr*103) and *unc*-6(*ev*400) single as well as in the double mutants the AC was less polarized than in the wild-type (Fig. 3G, suppl. Fig. s1B). Thus, loss of *madd*-2 function did not fully restore AC polarity in the absence of UNC-6 signaling. We also investigated whether the loss of AC polarization in *madd*-2(*tr*103) mutants affects the direction of invasion by observing the localization of HIM-4::GFP, which the AC normally secretes only ventrally toward the BL (Fig. 3H) (Sherwood et al., 2005; Vogel and Hedgecock, 2001). In *madd*-2(*tr*103) mutants, around 8% of total HIM-4::GFP protein was secreted on the dorsal side of the AC (Fig. 3I, suppl. Fig. s1C, D). Taken together, these results indicated that *madd*-2 is required to polarize the AC and direct its invasive machinery ventrally toward the BL.

Given the changes in AC shape and polarity, we hypothesized that the AC in *madd*-2(*tr*103) mutants might form ectopic protrusions, leading to an overall increased invasiveness. By observing the changes in AC shape with time-lapse microscopy, we found that the AC in *madd*-2(*tr*103) mutants was more dynamic than in the wild-type or in *unc*-6(*ev*400) single mutants, frequently forming ectopic protrusions on the dorsal or lateral plasma membranes (Fig. 4A–D, suppl. movies s1–4). Quantification of the recordings using an unbiased computer algorithm to measure AC shape changes (see Materials and methods section) confirmed the increased variability in AC shape, both in *madd*-2(*tr*103) single and *madd*-2(*tr*103); *unc*-6(*ev*400) double mutants (Fig. 4E). The formation of undirected, ectopic membrane protrusions could explain why loss of *madd*-2 function partially suppresses the basal lamina breaching defect of *unc*-6(*ev*400) mutants.

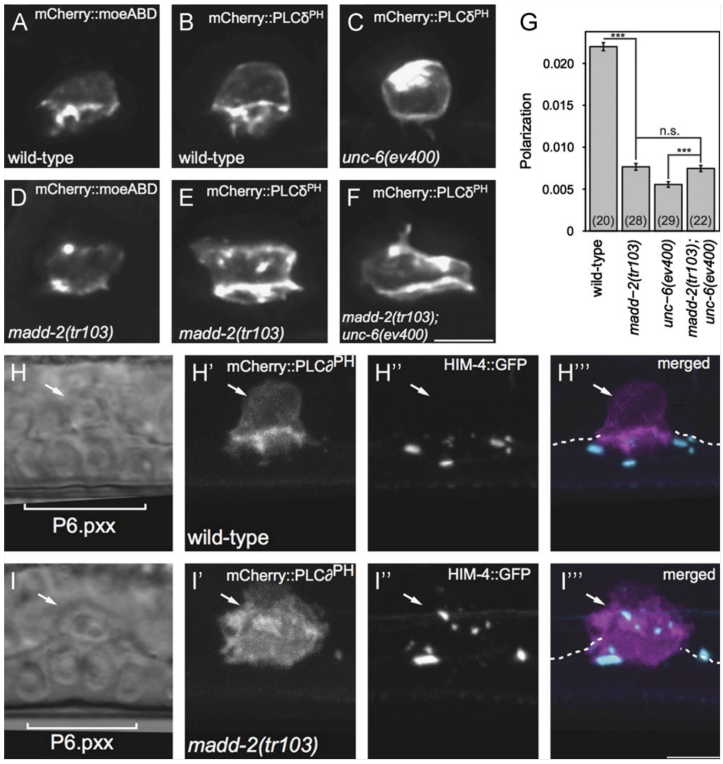

Fig. 3. MADD-2 regulates AC shape and polarity. (A through F) Maximum intensity z-projections of the AC expressing the actin marker mCherry::moeABD (*qyIs*50) (A,D) or the PIP_2 marker mCherry::PLCδ^{PH} (*qyIs*23) (B,C,E,F) in the indicated genotypes. (G) Quantification of AC polarity using the PIP_2 reporter as described in suppl. Fig. s1B. Error bars indicate the s.e.m. as estimated by bootstrapping and (n) the number of animals analyzed. p-values derived from bootstrapping are indicated with $p^{***} < 0.0001$ and $n.s. > 0.05$. (H) Nomarski image, (H′) maximum intensity z-projection of mCherry::PLCδ^{PH}, (H″) HIM-4::GFP and (H‴) merged images in the wild-type and (I through I‴) a *madd*-2(*tr*103) mutant at the Pn.pxx stage. The dashed lines in (H‴) and (I‴) indicate the BL, arrows the AC and the brackets the 1° vulva cells. Scale bars: 5 μm.

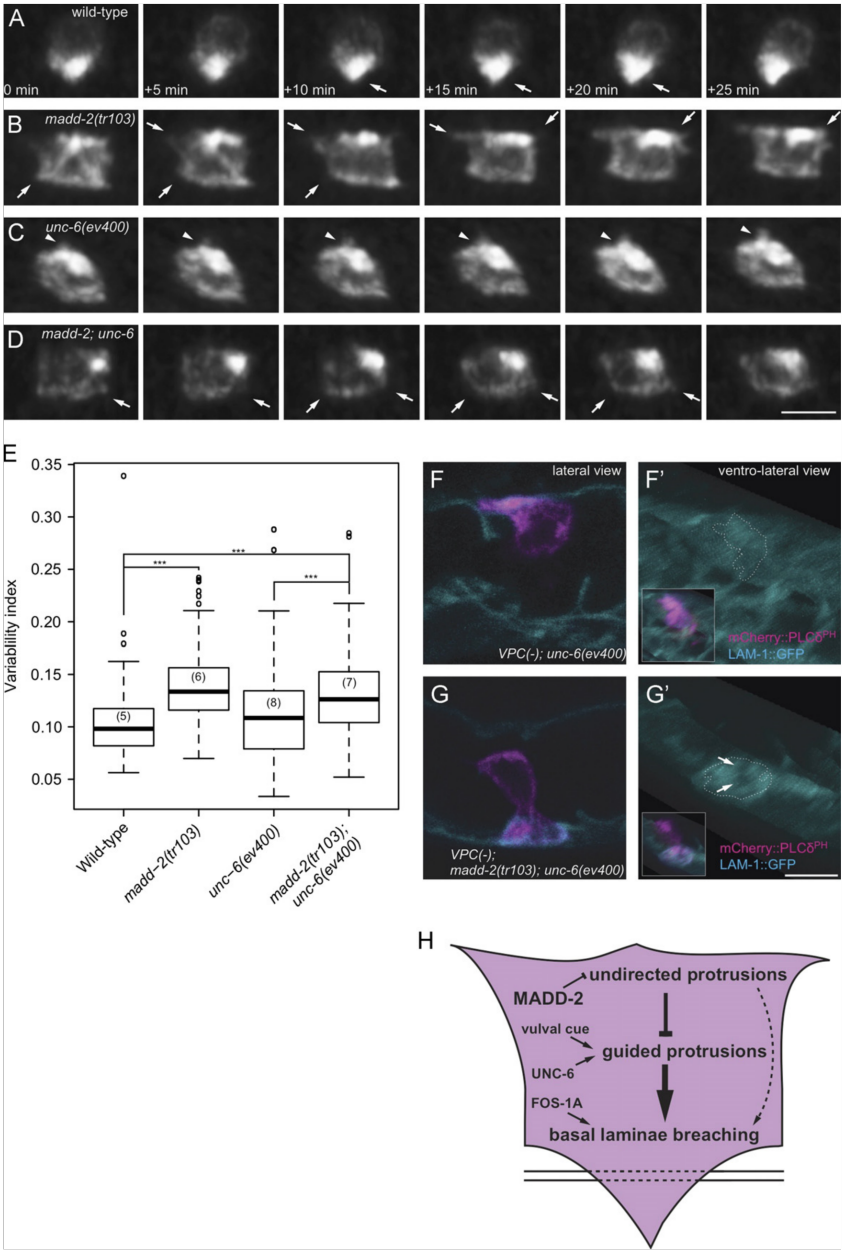

Fig. 4. Increased AC dynamics and unguided BL attachment in *madd-2* mutants. (A through D) Time-lapse recordings of the AC in the indicated genotypes visualized with the mCherry::PLCδ^{PH} marker (maximum intensity *z*-projections of lateral views, cropped *z*-stacks). Dynamic and static membrane protrusions are indicated with arrows and arrowheads, respectively. The BL is located ventrally, below the AC. (E) Quantification of AC dynamics. Box plots show the variability indices (ICQ) measured with the algorithm described in the Materials and methods section. An index of 0 indicates perfect overlap (i.e. no AC shape changes), and an index of 1 indicates no overlap (i.e. complete shape change). Hinges are drawn at the third and first quartile and the whiskers extend to the maximal value within 1.5-fold the box size from the box. (*n*) indicates the number of recordings analyzed. *p*-values derived from bootstrapping are indicated with $p^{***} < 0.0001$. (F) VPC ablation in an *unc-6*(*ev*400) single and (G) a *madd-2*(*tr*103); *unc-6*(*ev*400) double mutant. The ACs are labeled with mCherry::PLCδ^{PH} (magenta) and the BL with LAM-1::GFP (cyan). (F′,G′) show the corresponding ventro-lateral views of the BL. The insets show merged images to indicate the position of the AC and dashed lines delineate the region on the BL underneath the AC. Note the intact BL in (F′) and the arrows pointing at regions with reduced LAM-1::GFP staining in (G′). Scale bars: 5 μm. (H) Model for MADD-2 function during AC invasion.

Supplementary material related to this article can be found online at http://dx.doi.org/10.1016/j.ydbio.2012.11.019.

MADD-2 prevents AC invasion in the absence of any guidance signals

In addition to the UNC-6 Netrin signal from the VNC, the signal from the 1° vulval cells helps to guide the AC into the vulval tissue (Ziel et al., 2009). Only if the UNC-6 and vulval signals are simultaneously eliminated, AC invasion is completely blocked. We therefore tested if *madd*-2 prevents AC invasion in the absence of any guidance signals by ablating the six VPCs at the mid L2 stage in an *unc*-6(*ev*400) background. In *unc*-6(*ev*400) single mutants lacking the vulval cells, the AC was detached from the BL in 4 out of 10 cases and no signs of BL breaching were detected (Fig. 4F). However, in VPC ablated *madd*-2(*tr*103); *unc*-6(*ev*400) double mutants, the AC was attached to the BL in 8 out of 9 cases and in 3 cases also attempted to breach the BL, though we never observed a complete dissolution of the BL (Fig. 4G). Thus, even in the absence of any guidance signals the AC was attached to the BL in most *madd*-2(*tr*103) mutants.

Regulation of AC invasion by MADD-2

Based on our results, we propose that MADD-2 suppresses the formation of ectopic AC protrusions when no guidance signals are present. By inhibiting the intrinsic, invasive potential of the AC, MADD-2 sets a threshold for the formation of invasive membrane protrusions (Fig. 4H). As a potential ubiquitin ligase, MADD-2 may induce the degradation of pro-invasive factors such as regulators of actin polymerization. Once AC invasion is initiated, the UNC-6 and vulval guidance signals can locally overcome this inhibition and recruit the invasive machinery to the ventral side of the AC to form an invasive membrane on the BL. The ectopic protrusions formed in the absence of MADD-2 will compete with the formation of an invasive membrane and as a result reduce the overall efficiency of AC invasion. Thus, the invasion defect in *madd*-2 single mutants may be explained by an insufficient focussing of the invasive machinery toward a defined site on the BL over the vulval cells. This model also explains the partial rescue of the *unc*-6(*lf*) invasion phenotype by *madd*-2(*lf*), as the random protrusions formed in the absence of MADD-2 may increase the likelihood of BL breaching and thereby partially compensate for the loss of the Netrin guidance signal. Furthermore, our findings indicate a distinct role of MADD-2 in the AC compared to its previously described functions in muscle arm extension and axon guidance, where MADD-2 facilitates UNC-40 signaling (Alexander et al., 2010; Hao et al., 2010). Finally, the expression of the human *madd*-2 homolog *Mid*1 is significantly down-regulated in invasive melanoma cell lines, suggesting that the function of *madd*-2/*Mid*1 during cell invasion has been conserved (Berthier-Vergnes et al., 2011).

Taken together, we show that efficient cell invasion requires a precise balance between pro- and anti-invasive factors, allowing a cell to focus its invasive machinery to a defined region on the plasma membrane. In this context, MADD-2 is the first identified inhibitor of invasive membrane protrusions during AC invasion in *C. elegans*.

Materials and methods

General methods and strains used

Standard methods were used for maintenance of *C. elegans* (Brenner, 1974). *C. elegans* Bristol, variety N2, was used as wild-type reference. Mutations and transgenes used in this study: LGI: *unc-40*(*e*271), LGII: *qyIs*23[$P_{cdh\text{-}3}$::*mCherry*::$PLC\delta^{PH}$], LGIII: *trIs*31[*madd*-2::*gfp*], *rhIs*23[*him*-4::*gfp*], LGIV: *qyIs*10[*lam*-1::*gfp*], LGV: *madd*-2(*tr*103), *madd*-2(*ok*2226), *fos*-1(*ar*105)/*nT*1[*sqIs*51, *let-XX*(*m*435)](IV;V), *qyIs*50[$P_{cdh\text{-}3}$::*mCherry*::*moeABD*], LGX: *unc*-6 (*ev*400).

Microscopy and image analysis

Images were acquired using an Olympus BX61 microscope with a QImaging Retiga 2000R camera or a Leica DMRA wide-field microscope with a Hamamatsu ORCA-ER camera controlled by Openlab 5 (Improvision) and μManager (Edelstein et al., 2001). Confocal images were acquired using an Olympus FV1000 microscope. MitoTracker staining was performed as previously described (Rimann and Hajnal, 2007). Images were processed using Huygens Deconvolution (SVI) for *z*-stacks, Fiji (ImageJ (NIH), Imaris (Bitplane) and Adobe Photoshop. For time-lapse imaging, worms were embedded in 4% agarose containing approximately 5 mM tetramisole, covered by a coverslip and sealed with Halocarbon oil 700 (Sigma). Images were recorded on an Olympus BX61 wide-field microscope equipped with a DSU spinning disc unit at 5 min intervals and 0.3 μm *z*-spacing. For quantification of AC dynamics, at least 5 movies per genotype were deconvolved, maximum *z*-projected, edges calculated to enhance the contrast of the images (Image J plugin), and images were registered using the StackReg plugin (Image J) to correct for movements of the animals during acquisition. As a measure of AC dynamics, an intensity correlation coefficient (0.5-Li's ICQ) was calculated across the time frames and plotted in Fig. 4E (Li et al., 2004). This method allows us to score the temporal changes in AC shape in an automated and unbiased fashion using an algorithm that requires no user interaction. Quantification of the AC polarity, shape, dynamics and HIM-4::GFP deposition was done using custom written, semi-automated scripts in Image J and R applied to deconvolved wide-field or confocal *z*-stacks as described in suppl. Fig. s1.

Statistical analysis

Statistical analyses were performed by bootstrapping (Efron, 1981) using a custom written script for R (http://www.R-project.org). Briefly, the acquired data is resampled with replacement, creating many (10,000) bootstrap samples. As described by Efron, 1981, the standard deviation within these samples is an estimation of the standard error of the mean and the mean of these samples an estimation of the mean of the acquired data.

VPC ablations

VPCs were laser ablated in mid L2 larvae mounted on 3–4% agarose pads using a Leica DMLB wide-field microscope equipped with MicroPoint Laser system (Photonic Instruments).

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2012.11.019.

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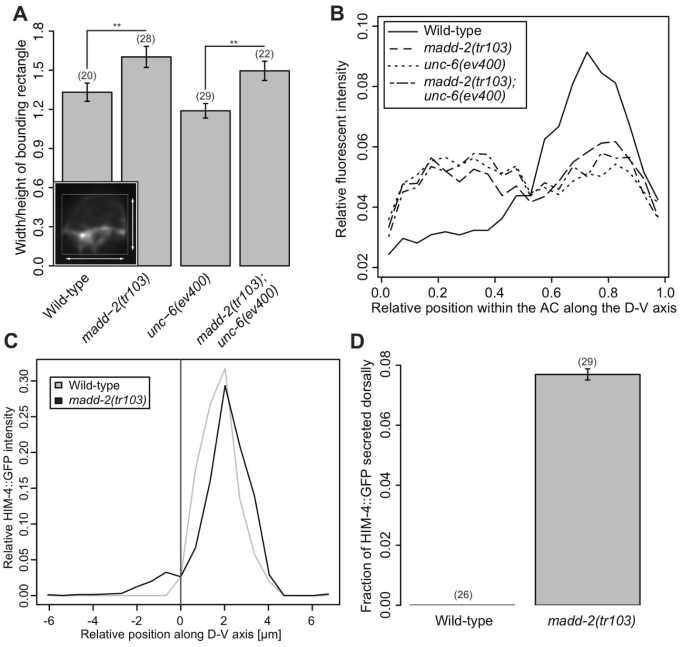
suppl. Figure s1



Supplementary Material

Suppl. Fig. s1 Quantifications of AC shape and polarity

(**A**) The AC width to height ratio was calculated using the ImageJ measurement tool to determine the bounding box on deconvolved, z-projected and thresholded wide-field z-stacks of the AC labelled with the mCherry::PLCδ^{PH} reporter, as indicated in the inset. (**B**) Polarity profiles showing an intensity histogram of the relative fluorescent intensity of the mCherry::PLCδ^{PH} reporter measured along the D-V axis on deconvolved, sum z-projected and thresholded wide-field z-stacks. On the x-axis, dorsal is left and ventral is right. For the quantification of AC polarization shown in Fig. 3G, the standard deviation of the polarity profiles along the y-axis were calculated, as a measure of how much the polarity profile varies along the D-V axis. (**C**) HIM-4::GFP intensity distribution along the D-V axis relative to the geometric center of the AC labelled with the mCherry::PLCδ^{PH} reporter. Confocal z-stacks were sum z-projected, thresholded and intensities were measured along the D-V axis. (**D**) Fraction of HIM-4::GFP secreted dorsally determined as shown in (**C**). Error bars indicate the s.e.m. as estimated by bootstrapping and (n) the number of animals analyzed. p-values derived from bootstrapping are indicated with **<0.001.

Suppl. movies s1-4 MADD-2 regulates AC dynamics

Time-lapse recordings of the AC labelled with the mCherry::PLCδ^{PH} reporter (z-projections) in (**movie s1**) wild-type, (**movie s2**) *madd-2(tr103)* , (**movie s3**) *unc-6(ev400)* and (**movie s4**) *madd-2(tr103); unc-6(ev400)* animals. The BL is located ventrally, below the AC. Z-stacks were recorded every 5 minutes as described in Materials and Methods. To create these movies -but not for the quantification shown in Fig. 4E- the deconvolved z-stacks were manually trimmed in the z-dimension.

7.3 Additional experiments and observations

7.3.1 AC invasion is regulated by basal lamina markers

One result that we did not mention in the publication was found by coincidence rather than by design. When scoring invasion in the various single and double mutant phenotypes, we used different methods to help visualize the basal laminae and to identify whether it was broken or intact. What we ended up using prevalently for the publication was MitoTracker, a live-tissue stain. Presumably, an added stain influences the invasion process as little as possible compared to transgenes that are part of the genetic background.

The results obtained with two of the three markers used, MitoTracker and LAM-1, showed the same relative degrees of invasion in *madd-2* mutants (Fig.7). But the absolute invasion frequency with the two markers is substantially different. A third marker, HIM-4, showed a different phenotype. It caused a significant increase in early invasion, but not later. In fact, the invasion frequency with HIM-4 as marker is comparable between the 2- and 4-cell stage.

We used two different fluorescently labelled transgenes, him-4 and lam-1, and the aforementioned fluorescent dye, MitoTracker. It could be reasonably expected that there is a difference between using transgenes or a dye as marker. Interestingly, there also was a difference between the two transgenes. In fact at the 2-cell stage of vulval development, using a labelled HIM-4 as marker for basal laminae integrity rescued invasion defects, while the LAM-1 transgene enhanced them. Although this was not necessarily expected, it can very well be explained. *him-4* is the one invasion executor gene with the strongest single mutant phenotype (although it still is not a very strong one) [38]. When it is expressed by a transgene, expression-levels are likely elevated compared to wild-type, resulting in enhanced invasion efficiency. Whether or not the invasion defect observed for the LAM-1 transgene is due to a similar function related reason or not is difficult to say. The simple presence of the transgene could retard AC invasion. To differentiate between the two possibilities, a comparison to a transgene inserted in the same genomic locus as the lam-1 transgene is necessary. A specific defect due to *lam-1* overexpression could result from increased basal laminae substance. This would require more effort to breach and a delayed invasion as consequence.

Importantly, the presence of any transgenic marker can alter the studied wild-type behaviour. Using different markers in parallel could help to verify results from experiments using transgenic markers. Also, when comparing different genotypes, the transgenic markers used should be identical. Not just because the marker itself can have an unknown phenotype, but because

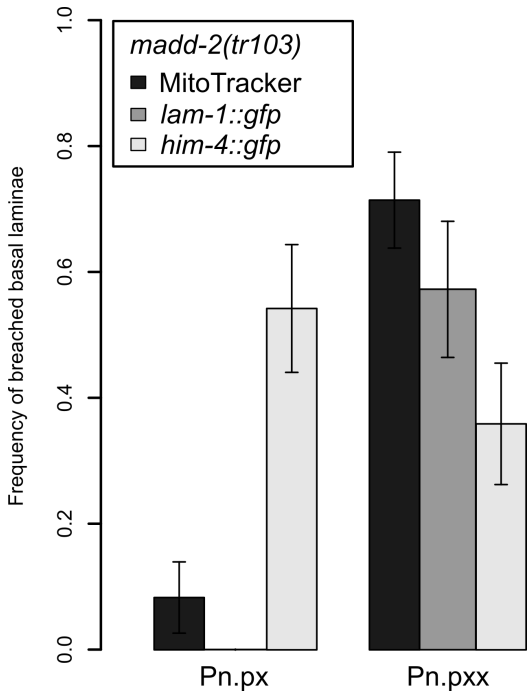


Figure 7: Transgenes affect the phenotype
Frequency of observed basal laminae breaching is shown at the 2- and 4-cell stages of vulval development in *madd-2(tr103)* mutants.

any transgene can be linked to additional, uncharacterized mutations.

7.3.2 K07D4.7, *ephx-1*, is a possible downstream target of *madd-2*

We wanted to understand not just what the role of *madd-2* during AC invasion is, but also the mechanisms behind the both repressive and activating invasion phenotype. We wanted to identify genes downstream of *madd-2* using a suppression screen based on the AC shape phenotype. We screened a small set of candidates that we selected based on what we thought causes the AC shape phenotype. We hypothesized that *madd-2* could downregulate actin regulators by uqiquitination through its E3 ligase activity. Consequently, the *madd-2* phenotype would result from increased actin polymerization. Actin polymerization is controlled on several levels, by Rac, Rho, and Cdc42 proteins [52]. These proteins control different aspect of actin dynamics and polymerization. Although Rho is more commonly implicated in actin depolymerization, all the different aspect and proteins are tightly interconnected in many different ways. Because we thought that *madd-2* mutants have overac-

tivated Rac, we knocked-down the *C. elegans* Rac homologues and the (predicted) GEFs of Rac/Rho proteins in a *madd-2* mutant background using RNAi. The knock-down of *ect-2*, a RhoGEF and activator of Rho signalling [82], transformed the AC shape similarly to *madd-2* mutants (Fig.8). This supported our hypothesis of enhanced actin polymerization in *madd-2* mutants. Molecularly, the *ect-2* knock-down likely inactivates Rho, which would effectively increase Rac activity. Based on the phenotypical similarity, the *madd-2* AC shape could thus be a consequence of overactivated Rac.

In the RNAi suppression screen testing around 20 different *C. elegans* Rac and RacGEF proteins, we found one candidate that restored the typical AC shape in a *madd-2* mutants. Notably, this phenotype reversal did not change the mutant AC polarity. The candidate was the predicted RhoGEF protein K07D4.7. K07D4.7, also called *ephx-1*, is the homologue of mammalian Ephexin. Ephexin is a predicted Rho-family GEF that can activate all three actin regulatory GTPases: Rho, Rac, and Cdc42 [83]. Both growth and collapse of axon growth cones is regulated by Ephexin [84]. This role of Ephexin in growth cone regulation is especially interesting, because axon guidance has a lot in common with AC invasion. Follow-up experiments however did not confirm *ephx-1* as a suppressor of *madd-2* for more than one reasons. The available *ephx-1* mutants did not reproduce the RNAi effects, the reporter never showed expression, and a role for *ephx-1* in muscle arm extension – performed by a collaborating group – was not found. We thus decided to focus instead on just the *madd-2* mutant phenotype alone and to characterize it in as much detail as possible instead of trying to find a downstream genes.

As a consequence, it is still not known how *madd-2* regulates AC invasion. One suggestion is that *madd-2* stabilizes *unc-40* [85]. In my opinion, the supporting evidence for this hypothesis is rather weak.

I do think that our approach to finding the *madd-2* mechanism was correct. However, we lacked the proper tools to complete the investigation. *ephx-1* is a very interesting candidate. The functions of its mammalian homologue are very suggestive when seen as a possible downstream component of *madd-2*. Most intriguingly perhaps is the association between the mammalian EphA2 receptor, its GEF Ephexin4, and an increase in tumor malignancy [86]. The fact the *ephx-1* mutant failed to corroborate the RNAi findings can have different reasons: the nature of the mutation in question could not eliminate gene function, the RNAi could have off-target effects, functional redundancy of *ephx-1* might prevent discovery of a phenotype using mutations. Nevertheless, even with all the potential technical difficulty, it might be worth having another look at *ephx-1* and the mechanism by which *madd-2* regulates AC invasion.

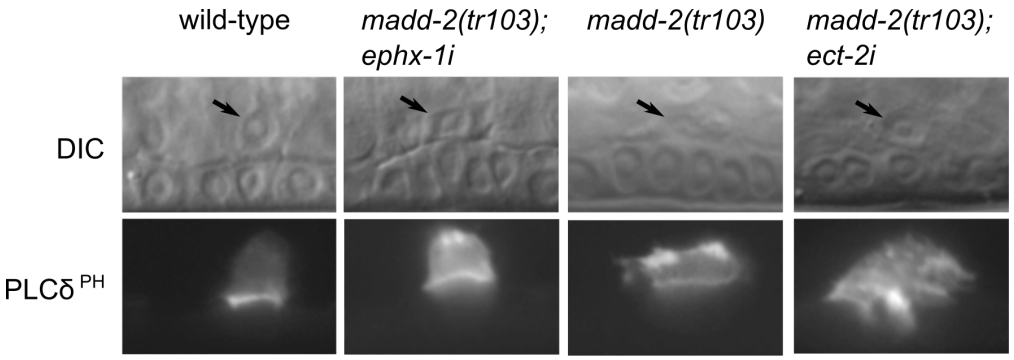


Figure 8: The *madd-2* AC phenotype can be suppressed and enhanced by manipulating actin regulators
DIC and fluorescent microscopy for the genotypes indicated. PLCδ^{PH}::mCherry is used to visualize the AC. *ephx-1i* and *ect-2i* refer to RNAi knock-down of *ephx-1* and *ect-2*, respectively. Images were taken at the early 4-cell stage of vulval development. The arrow points at the AC.

7.4 Conclusion

In this work, we described the role of *madd-2* in AC invasion in relation to mostly the *unc-6/unc-40* guidance system. We showed that *madd-2* regulates invasion upstream or in parallel to both *unc-6* and *unc-40* and separately from regulation of basal lamina breaching by *fos-1*. A very interesting aspect of the *madd-2* AC invasion phenotype is that it seems to act both as suppressor and enhancer of AC invasion. What we think is the reason for that is that *madd-2* mutants have a given level of AC invasion which is, to a certain degree, independent of the genetic background. So in an otherwise wild-type background, a *madd-2* mutation causes invasion defects, and in an *unc-6* or *unc-40* mutant, that already has invasion defects, it seemingly rescues this defect to some degree. What we think happens in fact is that the defect is just a strong as in the wild-type situation and that it ignores the *unc-6/Netrin* mutant background. This means that the apparent rescue of the *unc-6/unc-40* mutant is in fact a defect in the wild-type. For that reason, we suggest that *madd-2* regulates a separate pathway that stimulates undirected invasion. This pathway we propose overrides any guidance signal from the *unc-6/unc-40* cue and from the second cue too, so it does not matter whether the *unc-6* gene is wild-type or mutant. To try and illustrate this point, we checked the *madd-2* mutant phenotype in a guidance free background, with a mutation in *unc-6* and with ablated vulval tissue. The resulting phenotype suggested a remaining, unguided invasive activity,

even though proper basal lamina breaching was not observed. Nevertheless, compared to *madd-2* wild-type animals without guidance, there seemed to be an increase in invasion.

We only looked at the AC invasion phenotype during the study and as part of my thesis. However, there are still two other, also vulva/uterus related phenotype that we did not look at in detail. These are the developmentally earliest phenotype of AC duplication and the developmentally latest phenotype of π cell fate specification. I did not do any work related to the π fate specification, however, I made some preliminary observations that might be related to the AC duplication phenotype in *madd-2* mutants. Essentially, I was interested to know whether the AC duplication might be due to AC/VU precursor positioning or whether the reason was to be looked for somewhere else, possibly in compromised LIN-12/Notch signalling. The second possibility seemed the more likely one, because *madd-2* was originally found in a screen designed to find regulators of LIN-12/Notch signalling. To potentially cover both anticipated results, I checked a translational LIN-12/Notch reporter in mid-L2 larvae, before the AC/VU decision has been made. In few animals I could observe an apparent precursor mispositioning. Because the LIN-12/Notch ligand is membrane bound, the AC/VU precursors need to be in close spatial proximity in order for signalling to occur. In wild-type, they sit ventrally, in the middle of the developing somatic gonad. I found however animals in which the AC/VU precursors were displaced from one another. It happened that one of the precursors could be found dorsally and anteriorly displaced. However, I did not correlate misplaced AC/VU precursors with AC duplication, neither directly by checking the animals later in development again, nor indirectly via comparison of penetrance. This preliminary observation does however offer an explanation for the AC duplication phenotype and a possible way of attributing both AC duplication and invasion phenotype to the same mechanism, unguided invasion, or in the case of the duplication, migration. But as it is not clear from the literature how the AC/VU precursors do in fact assume their position in a wild-type situation, whether it involves migration or only proper orientation of the cell division axis, it is difficult to speculate on what contributes to the apparent mispositioning.

To purely speculate about the last phenotype, π cell fate specification, and especially whether also this phenotype could be related to enhanced cell migration and mispositioning, might be quite interesting. The uterine π fate is specified via LIN-12/Notch signalling from the AC. From where the AC sits, it sends the signal to the ventral uterine cells that it reaches. In the wild-type, the AC is centred amidst the future π cells. Consequentially, it is not hard to imagine a situation where the AC is out of position and thus does

not reach all the cells that it is supposed to. The possibility of the VU cells being out of position is rather unlikely, on the other hand, since both ventral and dorsal uterus are rather tightly packed and cell movement most likely restricted. However, I cannot comment on the positioning of any cells other than the AC, be it in the L2 stage or later. So theoretically, also the third and last of the known vulval *madd-2* mutant phenotypes could be explained by excessive migratory behaviour, leading to a mispositioned AC.

Overall, the *madd-2* mutant phenotype clearly demonstrates the importance of exact AC positioning and the multiple effects a relatively minor things such as being even slightly out of position can have on the development of even a simple organism such as *C. elegans*, and in particular on such simple organs like uterus and vulva, as is also shown in part two of this thesis.

Interestingly, this might be a case where development in the simple *C. elegans* is more sensitive to alterations, because it is only one cell that induces the different cell fates, as opposed to higher organisms where this kind of function is usually fulfilled by a small group of cells instead.

8 Part 2 – AC polarity negatively regulates vulval induction

8.1 Introduction

Epithelial cells are polarized. Generally, one differentiates between the apical and basolateral portion of polarized cells. The apical side is the one that faces a lumen, the basolateral one makes contact with neighbouring cells and the basal lamina/extracellular matrix. The two polarity compartments are separated by a set of junctional proteins. These not only form a tight, quasi-impenetrable connection to the neighbouring epithelial cells but also isolate the apical from the basolateral membrane. Establishing and maintaining cell polarity is tightly linked to secretion. Secretion, like polarity, differentiates between the apical and the basolateral membrane compartments. Which cargo is transported by which branch of the secretion machinery is mostly determined by signals encoded in the cargo itself. For basolaterally targeted proteins, it is well characterized signals in the C-terminal portion of the amino acid chain. Apical sorting signals are much more diverse, on the other hand, and can also be encoded not through amino acid motifs but for example by clustering into lipid rafts or glycosylation [87].

Cell polarity pathways and their establishment have mostly been studied in Madin-Darby canine kidney (MDCK) cells *in vitro*. These cells apparently have an innate ability to polarize easily and were thus extensively used to

study any and all kinds of polarity related pathways and phenotypes *in vitro*. Another, slightly less popular model, are retinal pigment epithelium (RPE) cells. These are human cells that line the retina and stimulate the photoreceptor cells in various ways, including prevention of vascularisation and cell senescence. In these and other models, the secretion of many different growth factors, TGF-α and TGF-β, PDEF, PEDF, VEGF and others have been studied. Some of these are secreted in a polarized fashion, whilst others aren't, depending on cell type and/or model. It seems most growth factors that are secreted preferentially, either apically or basolaterally, simply take either the apical or the basolateral route to the membrane, respectively, and have a corresponding signal in their protein [88]. Of interest might be the finding that polarity correlates positively with expression for some growth factors in RPE cells. Upon polarization of the cells *in vitro*, expression of a growth factor can be boosted severely, whilst maintaining a polar secretion profile [89].

The cell and growth factor in question in this thesis are the AC and LIN-3/EGF. The AC is clearly polarized, however the naming of the polarity can be a bit confusing. Typically, the apical side faces a lumen and the basolateral one other cells and/or the extracellular matrix. The AC on one side faces the extracellular matrix/basal lamina and further the vulva and on the other the dorsal uterine cells. But eventually, both sides will face a lumen. The vulval lumen on one side, where the basal lamina is in the beginning, and the uterine lumen on the other, where the dorsal uterine cells are at the start. By the lumen based definition of apical, the AC is thus a cell that has two apical sides. Another way to name the membrane compartments is according to junctions. Also here, the AC has more than just the typical, single ring of junctional proteins [31]. By all means, the AC is a very special polarized cell, but highly polarized nonetheless.

The question remains how and whether the AC secretes LIN-3/EGF in a polarized fashion during vulval induction. The textbook model, for seemingly no other reason than common conception, states that LIN-3/EGF forms a gradient, originating from the AC. Gradients are indeed a widespread theme in the release of growth factors for several reasons. Assuming random diffusion of a growth factor after release from the source, a concentration gradient will form naturally. Also, gradients permit growth factors and especially morphogens to induce different cell fates depending on the distance of the target cell from the source, and guidance along a gradient is easy in principle. For all these reasons, gradients have been widely proclaimed and serve as a great model to explain many phenomenons. In many cases, gradients have also been visualized, although this specifically is not trivial, mostly due to technical difficulties and the high sensitivity necessary for detection.

Despite the all present notion of gradients, alternative theories have been postulated. The most famous one is probably that of cytonemes [90]. Cytonemes are a structure that the growth factor/morphogen producing cell extends towards the receiving cells. They are very thin, filopodia like protrusions that deliver the growth factors and morphogens in a directed and processive manner to their target. In some instances, there is evidence pointing towards the existence of such cytonemes, e.g. for hedgehog or Dpp signalling in *Drosophila*.

8.2 LIN-3/EGF

Whether there is a LIN-3/EGF gradient or not, LIN-3/EGF reaches first and foremost P6.p and to a lesser amount the P6.p neighbours P5.p and P7.p. This is based on the expression of *egl-17* [67]. *egl-17* is a direct target of the LET-60/Ras pathway and its expression is strongest in P6.p, strongly reduced in P5.p and P7.p and absent from the other VPCs. Also, LET-23, which is a target of LET-60 too, is very strong in P6.p compared to the other VPCs [91]. A direct visualization of LIN-3 and its distribution is difficult. This could be due to the small distance between AC and vulval epithelium, an unfavourable environment for fluorophors, or rapid turnover of the molecule. But based on the observations of LIN-3/EGF targets, a potential gradient of LIN-3/EGF is expected to be very narrow.

8.2.1 Different factors influence the distribution of LIN-3/EGF

How crucial is AC derived LIN-3/EGF distribution really? Assuming a wild-type situation where LIN-3/EGF is directly released toward P6.p, 100% of induction-relevant LIN-3/EGF would be absorbed by the presumptive 1° cell (Fig.9). In this situation, the lateral inhibitory Notch signal would be of no importance. However, it would be strongly expressed and is obviously still required for induction of the 2° signal. In the other extreme, LIN-3/EGF would be randomly and uniformly released from the AC. The AC still is closest to P6.p and thus, approximately 25% of AC derived LIN-3/EGF can reasonably be expected to be taken up by P6.p. Both P5.p and P7.p would likely receive only about half that amount, possibly less. Combined with the inhibition mechanism via LIN-12/Notch, this would arguably still be enough to attain a wild-type vulval cell fate pattern. And indeed, no polarity genes have been found to affect vulval cell fate in a penetrant fashion. Only very seldomly do mutants in *unc-6* result in altered vulval induction (Fig.13). The question might thus be: Is the polarity of growth factor release really important? The vulval induction machinery has evolved to be able to

compensate variations in the signals necessary to form a vulva. There are several compensatory mechanisms at work, presumably because under non-laboratory conditions, induction based on one system alone would not be reliable enough. In other words, the capacity to compensate for fluctuation is necessary, and it is very likely that LIN-3/EGF polarity is one of them, based on what can happen when wild-type LIN-3/EGF distribution patterns are disrupted.

In the following, I will cover a selection of factors that affect LIN-3/EGF distrubtion in more detail.

dig-1 – Misplacing the gonad with the AC

dig-1 mutants are interesting because in some cases, the whole gonad lies dorsally instead of ventrally just atop the vulval tissue. This increases the distance from the inductive AC to the induced vulval tissue. A vulva is still formed in most cases though, indicating the diffusible nature of LIN-3/EGF [68]. The pattern, however, is lost. It is in fact hard to try to make sense of the cell fate pattern at all, taking into account the current model of vulval induction. The two extremes are exclusive 2° cell fate and isolated 1° cell pate. Both are probably a consequence of low levels of LIN-3/EGF reaching the VPCs. It has been claimed that low levels of LIN-3/EGF can induce 2° cell fate [58]. Alternatively, equal low levels of LIN-3/EGF might stimulate LIN-12/Notch signalling in all VPCs and reciprocal stimulation could make all cells 2°, because the low LIN-3/EGF signal, that promotes the 1° fate, would be overridden by LIN-12/Notch. The isolated 1° fate could result from even lower LIN-3/EGF levels, not even strong enough to induce the lateral LIN-12/Notch signal. It seems that *dig-1* not only reduces the gradient, but also the dosage.

As a consequence, trying to conclude the importance of the gradient is more difficult, because also the dosage is varied. Overall though, losing the LIN-3/EGF gradient seems to equalize the LIN-3/EGF levels across the VPCs, which in turn might affect the function of the lateral inhibitory mechanism.

let-23(sy1) – Misplacing the LIN-3/EGF receptor LET-23

LET-23 mislocalization also changes the distribution of LIN-3/EGF. This has been illustrated using a *gap-1* mutant (Fig.9)[25]. LET-23 binds LIN-3/EGF, internalizes it, and signals to the downstream pathway components. Because LIN-3/EGF is internalized, it is essentially single-use only. In other

words, signal strength and free LIN-3/EGF concentration are inversely correlated. On the other hand, when the signal is reduced due to changes in receptor concentration, there will be more extracellular LIN-3/EGF compared to the wild-type situation. This is the siutation if LET-23 is absent from the basolateral VPC surfaces, due to a knock-out or mislocalization. As a consequence of missing LET-23, LIN-3/EGF concentration is higher and the gradient wider. In an otherwise wild-type background, the lack of signalling LET-23 due to mislocalization results in a severe vulvaless mutant, with almost no cells induced at all. However, even if LET-23 is mislocalized, it still signals at low levels. This is because LET-23 briefly appears at the basolateral surface of the VPCs before it traffics to the apical side in a *let-23(sy1)* mutant. In combination with a mutation in *gap-1*, which amplifies signalling of the LET-23/LET-60 pathway, the vulvaless *let-23(sy1)* mutant is transformed into a moderately strong multivulva double mutant, with between four and five cells induced. LIN-3/EGF now reaches cells that it would not reach in a wild-type background, and the low levels of inductive signal are amplified. Together, these two factors lead to more than just three cells adopting a vulval cell fate.

However, the question what influence the LIN-3/EGF distribution has on vulval induction in a wild-type situation is difficult to answer based on these observations. *let-23(sy1)* mutants are vulvaless and together with *gap-1* become multivulva. So even though LIN-3/EGF distribution is altered in this situation, drawing conclusions regarding the wild-type is difficult.

lin-3 overexpression – Misplacing LIN-3/EGF itself

Another possible way to influence LIN-3/EGF distribution is overexpression. Any gradient can be overridden with high levels of exogenously expressed protein. If *lin-3* is expressed from a heat-shock promoter in *C. elegans*, any cell can become a source of LIN-3/EGF. Interestingly, it is possible to control the amount of additional LIN-3/EGF by varying the number of transgene copies and the strength of transgene induction, in this case by varying duration and temperature of the heat-shock, as done by Katz *et al.* [58]. In these experiments, the gonad was removed to eliminate endogenous LIN-3/EGF production. When LIN-3/EGF is expressed at low levels, all induced cells adopt the 2° cell fate. Even single induced cells would adopt a 2° cell fate. This is not readily agreeing with the sequential model of vulval induction that predicts that Notch determines the 2° fate. However, assuming that different levels of LET-23/LET-60 signalling are required to induce lateral LIN-12/Notch signalling (lower levels) and 1° cell fate acqui-

sition (higher levels), these findings can be explained easily. An additional mechanism at work is autocrine LIN-12/Notch signalling, that can override a weak LIN-3/EGF signal ([92]). If the dosage of heat-shock promoter driven LIN-3/EGF is such that it is sufficient for the induction of 1° cell fates, then the expected 1°-2° pattern can be observed. Only when LIN-3/EGF is heavily overexpressed, the adjacent 1° fate emerges.

Again, however, these experiments do not answer whether LIN-3/EGF distribution from the AC, a point source as opposed to the ubiquitous expression in this set of experiments, influences vulval cell fate pattern acquisition. Instead, these findings highlight the difference between very low and normal levels of LIN-3/EGF overall and might hint at different levels of competence among the VPCs.

8.2.2 The connection between LIN-3/EGF distribution and vulval induction

Polar secretion and/or trafficking of growth factors seems usually based on the normal apical and basolateral secretion mechanisms ([87]). This works well for normal epithelia with well defined apical and basolateral sides. In the AC of *C. elegans*, the standard epithelial concept is only partially applicable, as I explained above.

The AC is the source of the growth factor and potential morphogen LIN-3/EGF. LIN-3/EGF determines and induces 1° vulval cell fate. The VPC receiving the highest dose will form the future center of the vulva. It is important that LIN-3/EGF reaches the VPCs, as is illustrated by the phenotype of LET-23 mislocalization. If the LIN-3/EGF receptor LET-23 does not perceive the LIN-3/EGF signal, e.g. because it is mislocalized, vulval induction is severely reduced. Clearly thus, polarity of the receptor important. Ligand polarity on the other hand is assumed not to be important due to diffusion compensating for possible initial depolarization, but in fact it has been largely ignored.

8.3 Manuscript draft

This is a manuscript draft for the studies that form the second part of my thesis. It is currently incomplete because a few key experiments are not yet done. As a consequence, all the following is subject to change, including also the author list.

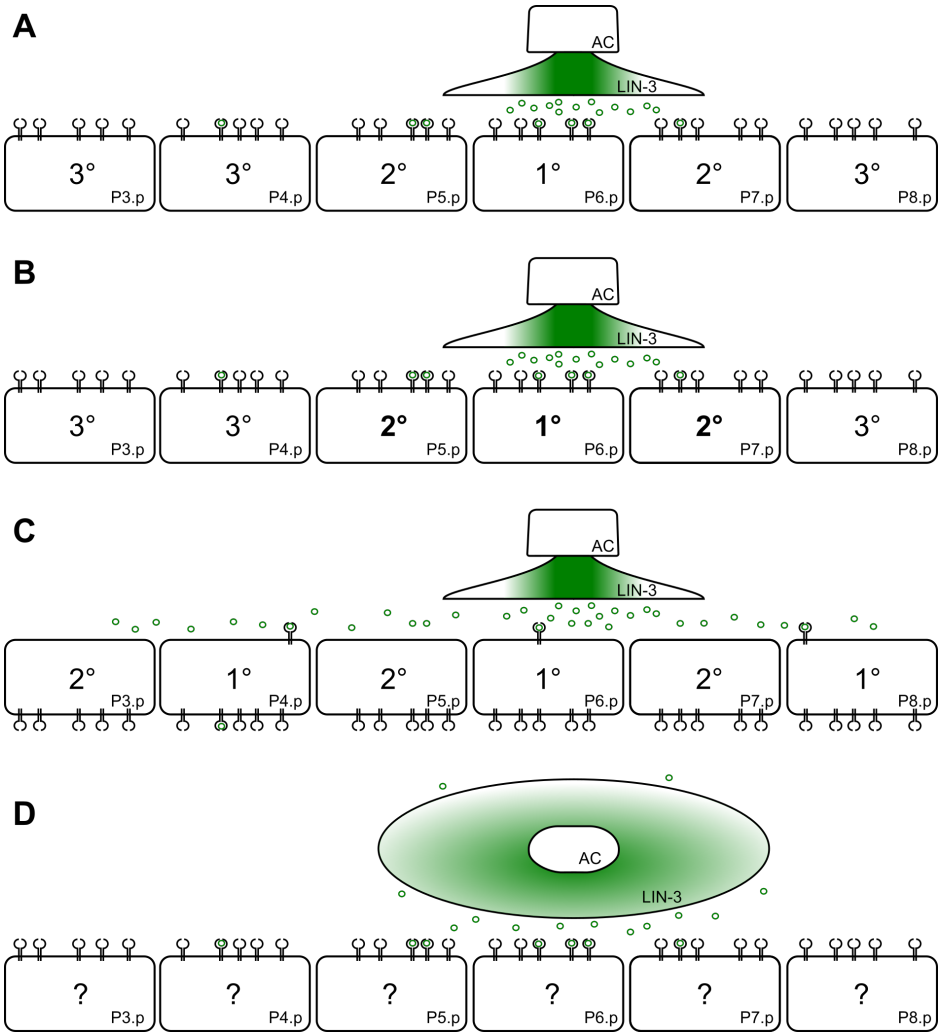


Figure 9: LIN-3 distribution and vulval induction
Depending on how LIN-3 is distributed, vulval induction is modfied. (A) is the wild-type situation. (B) shows the effect of lack of *gap-1*. Note the bold cell fates, meaning increased signalling. (C) illustrates the situation when *gap-1* is mutated and LIN-3 distributed more broadly, as in *let-23(sy1)* mutants. (D) is the hypothesized situation, in which LIN-3 distribution is altered due to AC polarity and not receptor availability.

LIN-3/EGF polarity limits vulval induction in *Caenorhabditis elegans*

Authors: Matthias K. Morf, Juan M. Escobar-Restrepo, Peter Gutierrez, Michael Daube, and Alex Hajnal

8.3.1 Introduction

Gradients have been a very successful and popular concept in devlopmental biology for a long time. And quite aptly so, they do indeed derive naturally from the notion of a point source and free diffusion. Also there is much evidence for their presence *in vivo* ([93, 94, 95, **?**]).

Morphogens are molecules acting concentration-dependently to induce different cell fates. Typically, morphogens act cell non-autonomously. The main view of morphogen activity involves secretion from the producing cell, passive diffusion and eventual sensing of the morphogen by cells competent to respond to morphogen presence by altering their cell fate. An opposing view postulates the existence of so-called cytonemes, long and thin cellular protrusion, similar to filopodia [90]. These cytonemes traffic morphogens in a directed manner.

Morphogen gradients take a very prominent role in assigning different cell fates to a uniform group of competent cells. Varying morphogen concentration, usually due to different distances from the secreting cell, elicit a concentration dependent response in the receiving cells.

During vulval induction in *C. elegans*, three cells of a group of six competent cells called vulval precursor cells (VPCs) are selected and induced to form the vulva [29]. The VPCs are called P3.p, P4.p, etc. until P8.p and in a wild-type situation, P5.p, P6.p and P7.p will always adopt the vulval cell fate. A specialized uterine cell, referred to as anchor cell (AC), secretes the EGF homologue LIN-3. LIN-3 induces the vulval cell fate. P6.p is the cell closest to the AC and receives the highest dose and thus adopts the 1° vulval cell fate. P5.p and P7.p receive then a lateral inhibitory signal from P6.p via the Notch/LIN-12 pathway and thus adopt the 2° vulval fate. The other three VPCs do not receive enough of either LIN-3 or LIN-12 and so adopt the non-vulval 3° fate.

LIN-3 secreted from the AC during induction has been assumed to form a gradient for a long time. In fact, there are a number of observations that do suggest such a graded distribution of LIN-3 does indeed exist. For example, an increased distance between source (AC) and the receiving cells (VPCs), as can be found in *dig-1* mutants with a dorsally misplaced uterus, do show a broader, aberrant pattern of induction compared to wild-type animals [68]. Similarly, *lin-3* overexpression induces additional vulval cells,

again broadening the reach of the *lin-3* signal [58]. Also, *egl-17* expression is graded, with the highest levels in P6.p and lower in P5.p and P7.p. [96].

This does indeed suggest the presence of a LIN-3 gradient being present, originating from the AC.

However, how this gradient might be established and whether its presence is indeed important for induction has never been addressed, other than the finding that LET-23 is crucial in restricting the diffusion of LIN-3 to limit vulval induction [25]. When LET-23 is mislocalized, it no longer sequesters LIN-3 and allows it to spread along the VPCs. In combination with a *gap-1* mutation that enhances the inductive signal during vulval development, this induces additional cells to adopt the vulval cell fate. This illustrates that the distribution of LIN-3 is important in controlling vulval induction.

Cell polarity is most clearly present in epithelial cells. These cells have an apical side, facing the lumen, a basolateral side, and a barrier in between that also connects to neighbouring cells via specialized junctions. The two sides are very distinct in terms of proteins present and diffusion in between is limited. Polarity can be found in other cell types too. Essentially any migratory cell is polarized into a leading edge and a trailing part, again characterized by different protein compositions. In the case of migratory cells, polarization is essential for processive movement.

An important feature and prerequisite for cell polarity is polarized transport [97]. There is apical and basolateral vesicle transport in polarized epithelia. Basolaterally destined cargo usually carries an intracellular sorting signal, at least the studied membrane proteins. Apical sorting signals can be much more diverse in nature, ranging from extracellular domains, to glycosylation to lipid raft association.

The AC is specialized uterine cell. It is also an epithelial cell and as such polarized. However, it is a very special cell in term of its polarity characteristics. A number of markers, as well as the cellular environment, define apical versus basolateral compartments in polarized epithelia. The side of the cell facing the basement membrane typically is the basal side. Conversely, the apical side is oriented towards the lumen. Apical junction molecules do, as their name suggests, lie at the boundary between apical and basolateral part. Phosphatidylinositol-(4,5)-bisphosphate is typically enriched at the inner leaflet of the apical side and PAR-3 is usually found at the apical side as well [98]. Visually, the AC will towards the end of its lifetime in the fourth larval stage, end up facing two different lumen, the vulval and the uterine. This would mean it has two apical sides. Also, in the third larval stage around vulval induction, the side that faces the basal lamina, so conventionally the basolateral side, is enriched for PIP_2, an apical marker. The junction molecules do cluster away from the basal lamina and support the

classical notion of apical, but PAR-3 does spread over the junctions and also cover what can only be referred to as lateral sides [31].

During the course of vulval development, the AC has a special task, which is to breach the tissue barrier between uterus and vulva in a process referred to as AC invasion. To do so, polarity is important in two ways: To know where to migrate to, and to dissolve the barrier [38, 31]. The guidance aspect is clearly demonstrated by displacing the target vulval cells so that the AC needs to form a long protrusion to reach it. If the polarity mechanisms are mutated, the AC does not even attempt to form protrusions, even in a wild-type setting, illustrating that there is indeed a guidance mechanism working. The need of polarity for breaching is not as clear, but can be inferred by the fact that there aren't any mutants that affect polarity without also affecting breaching. It is also demonstrated by the need of *unc-40* for both sensing direction for migration and for bundling the breaching efforts to allow efficiency [99].

Here, we study how the other developmental process that the AC is tightly involved in, vulval induction. In a screen for the mechanisms that control the localization of LET-23 and thereby vulval induction, we found that not only the polarity of the VPCs, but that of the AC is important in ensuring wild-type vulval induction. We ask whether and how the highly polarized nature of the AC affects the secretion of LIN-3 and present a possible mechanism thereof.

8.3.2 LIN-3 distribution is polarized

AC polarity is well established in the context of AC invasion [31]. But already at the beginning of the L3 stage, in the 1 cell stage of vulval development, is the AC heavily polarised (Fig.10). The polarity mostly affects cytoskeleton related molecules, such as actin, integrins and their regulators and adaptors [81]. We wanted to know whether LIN-3, the growth factor responsible for vulval induction, is polarised, especially during vulval induction. For that purpose, we made a LIN-3 translational reporter of which we integrated both multicopy and single copy transgenes, via X-ray and MosCI respectively. As expected, AC expressed LIN-3 is polarised towards the vulva during the early L3 stage (Fig.11), even though it is less than the actin associated PIP_2 marker PLCδ (Fig.12).

8.3.3 *unc-6* controls LIN-3 polarity

To investigate the effect of *unc-6* on vulval induction, we examined the distribution of LIN-3 within the AC during vulval induction at the beginning

Gene ID	Pvl	Muv	LIN-3::GFP	AC polarity
acbp-3	weak	weak	apolar	disturbed
acr-3	none	weak	-	-
cah-4	strong	weak	apolar	-
cav-2	weak	weak	apolar	-
cdk-2	weak	strong	intracellular	-
cdk-4	weak	weak	-	-
cki-1	strong	weak	intracellular	-
21ur-13219;clec-197	weak	weak	intracellular	-
cye-1	none	strong	-	-
dig-1	strong	weak	intracellular	-
din-1	strong	weak	depolarized	-
dpy-27	weak	weak	intracellular	disturbed
gei-17	weak	weak	-	-
hgo-1	strong	weak	intracellular	-
his-72;WBGene00013563	none	weak	-	-
hmg-3	none	strong	-	-
hpo-11	strong	strong	-	-
lact-9;WBGene00012885	weak	weak	intracellular	-
lag-2	strong	strong	-	-
lin-1	weak	strong	intracellular	-
lin-9	strong	strong	-	-
lin-14	strong	strong	mispolarized	-
madd-2	weak	weak	-	-
mca-1	weak	strong	intracellular	-
mes-6	strong	weak	-	-
nhr-103	strong	weak	-	-
nhr-115	weak	weak	-	-
nlp-26	none	weak	apolar	intact
puf-8	none	strong	intracellular	-
sdc-2	strong	weak	-	-
sem-4	strong	weak	intracellular	-
sra-9	strong	weak	intracellular	intact
srd-36	weak	weak	-	-
srh-8	strong	weak	-	-
srh-247	strong	weak	apolar	intact
toe-2	strong	weak	intracellular	-
ugt-14	weak	weak	-	-
ulp-1	weak	weak	-	disturbed
unc-40	strong	weak	intracellular	-
usp-48	strong	weak	intracellular	disturbed
WBGene00011820	weak	weak	apolar	-
WBGene00013481	weak	weak	-	-
WBGene00014082	weak	weak	-	-
WBGene00015203	strong	strong	-	-
WBGene00017836	none	weak	-	-
WBGene00020617	weak	weak	-	disturbed
WBGene00020696	weak	weak	apolar	-
WBGene00020960	none	weak	-	-
WBGene00044777	weak	weak	apolar	intact

Table 1: The 49 primary candidates from the screen
The candidates are listed alphabetically. The screen phenotypes (Pvl and Muv) indicate the penetrance of the respective phenotype. The effect of knock-down on LIN-3 and AC polarity (evaluated by examining the PLCδ^{PH} transgene marking PIP_2) is also shown unless marked by a minus.
Validating the *dig-1* RNAi clone by sequencing was not possible.

Category	Gene Identifier
Transmembrane Receptor Signalling	*acr-3, unc-40*
Intracellular Signalling	*puf-8, sem-4, toe-2*
Transcription	*din-1, hmg-3, lin-1, lin-9, lin-14, mes-6, nhr-103, nhr-115, sdc-2*
Transport	*acbp-3, acr-3, lag-2, mca-1,* WBGene00011820
Transmembrane Ion Transport	*acr-3, mca-1*
Synaptic Membrane	*acr-3*
Membrane-Associated	*acr-3, cav-2, cdk-4, lag-2, mca-1, sra-9, srd-36, srh-8, srh-247, ugt-14, unc-40, WBGene00013563,* WBGene00014082, WBGene00017836, WBGene00020960, WBGene00044777
Axon Guidance	*madd-2, unc-40*
Cell Migration	*dig-1*
Intracellular Organelle	*cki-1, cye-1, din-1, hmg-3, lin-14, sdc-2, sem-4*
Morphogenesis	*cah-4, dpy-27, gei-17, ulp-1*
Cell Cycle	*cdk-2, cki-1, cye-1, lin-9*
Mixed	*21ur-13219;clec-197, hgo-1, his-72, hpo-11, lact-9;*WBGene00012885, *nlp-26, usp-48,* WBGene00015203, WBGene00013481, WBGene00020617, WBGene00020696

Table 2: A rough categorisation of the screen candidates
The screen candidates are assigned to one or more categories, including functional categories and cellular compartments. Several rare categories were collected under the mixed tag.

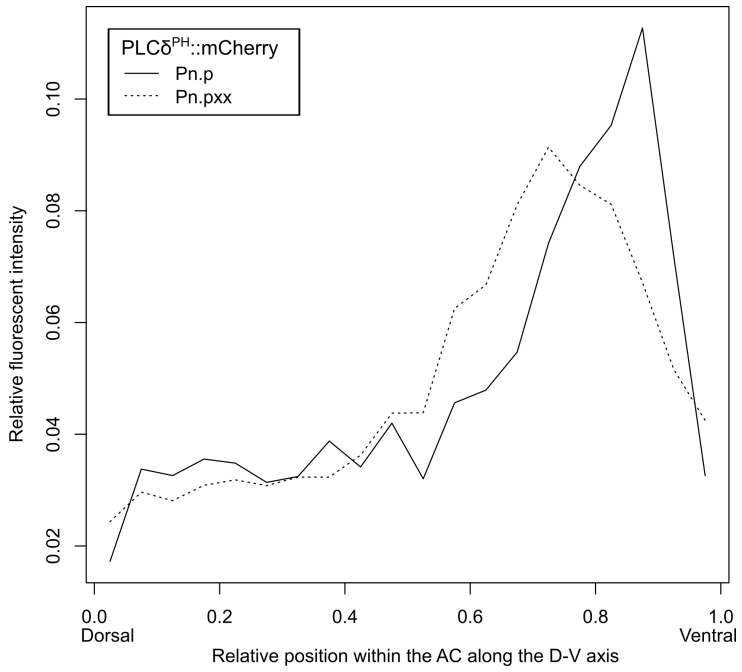


Figure 10: The AC is polarized before AC invasion
Polarity profile of PLCδ^{PH}::mCherry in the AC in wild-type worms at the beginning and middle of the L3 stage. The intensity histogram of the relative fluorescent intensity along the D-V axis of the AC was measured on deconvolved, sum z-projected and thresholded wide-field z-stacks. On the x-axis, dorsal is left and ventral is right. At least 20 animals were analyzed for both stages.

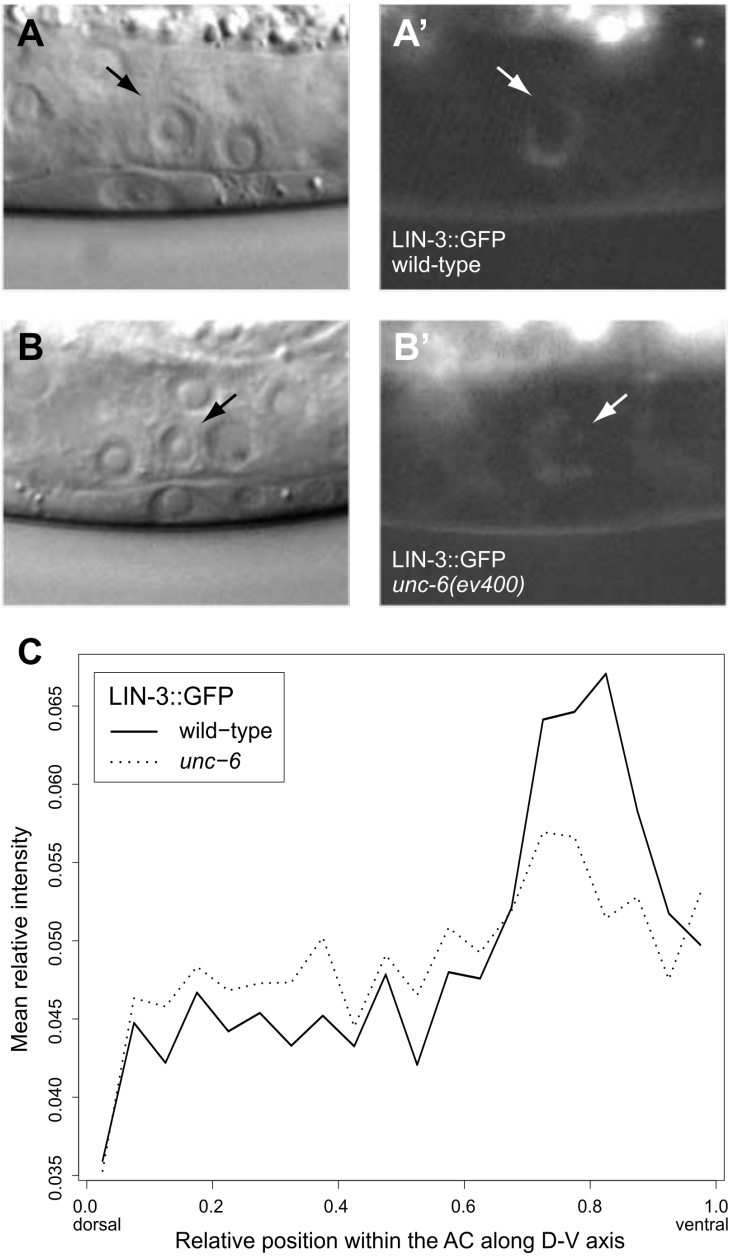


Figure 11: LIN-3 is polarized in the AC
DIC and fluorescent microscopy of the AC at the beginning of the L3 stage. (A,A') show wild-type, (B,B') *unc-6(ev400)* animals. The arrow indicates the AC. (C) is the quantification via intensity histogram of the relative fluorescent intensity along the D-V axis of the AC. Measurements were done on sum z-projected and thresholded wide-field z-stacks. On the x-axis, dorsal is left and ventral is right. At least 20 animals were analyzed for both genotypes.

of the third larval stage. We compared a single copy MosCI integration [8] of a GFP-tagged *lin-3* between wild-type worms and *unc-6* mutants, and found a significant difference. In wild-type, LIN-3 is clearly polarized towards the vulval epithelium (Fig.11A,A'). In *unc-6* mutants, the LIN-3 polarity is strongly reduced, if not abolished (Fig.11B,B'). In addition, the intensity of the single copy transgene seems reduced in *unc-6* mutants, in accordance with a broader distribution and lower concentration.

We hypothesize that this observed loss of LIN-3 polarity affects the LIN-3 distribution along the VPCs and the inductive pattern.

8.3.4 AC polarity modulates vulval induction strength

In a wild-type *C. elegans*, three of six potential cells get induced to adopt a vulval cell fate invariably. A mutation in the LET-60/Ras GAP *gap-1* enhances the strength of the inductive signal but not enough to cause a corresponding increase in the number of cells induced [25]. It thus serves as a sensitizing background to detect, among other things, a change in LIN-3 or LET-23 distribution. When combining mutations in *gap-1* and *unc-6*, the *C. elegans Netrin* homologue responsible for guiding the AC during invasion, vulval induction is clearly increased above wild-type levels (Fig.13A). Similarly, slightly weaker phenotypes result from combining *gap-1* with other AC polarity associated mutations, such as *unc-40* or *madd-2* (Fig.13B,C) [99, 100]. To try and rule out potential effects on other cells than the AC, we suppressed the *unc-40*, *gap-1* multivulva phenotype by expressing *unc-40* specifically in the AC (Fig.13C). This resulted in a slight rescue, but not complete suppression of the multivulva phenotype.

When combining an AC polarity mutant like *unc-6* with a mutation that decreases vulval induction, for example the strong loss of function of *lin-3*, it further enhances the vulvaless phenotype (Fig.13E). Combining *unc-40* with the weakly vulvaless *bar-1* mutation does not increase overall induction significantly, but leads to the appearances of completely vulvaless mutants not observed in the *bar-1* single mutant background.

As all the mutants displaying an AC polarity defect also have an invasion defect, we wanted to test whether it might be AC invasion, and not polarity, that affects vulval induction. We combined the AC invasion mutant *fos-1a(ar105)* with *gap-1(ga133)* and scored vulval induction. *gap-1* mutants show slightly elevated vulval induction compared to wild-type and the *fos-1; gap-1* double mutants increase induction even more. The additional increase is statistically significant, but based on data visualization not a qualitative difference (Fig.13F).

We would argue that, similar to the case of LET-23 mislocalization, AC

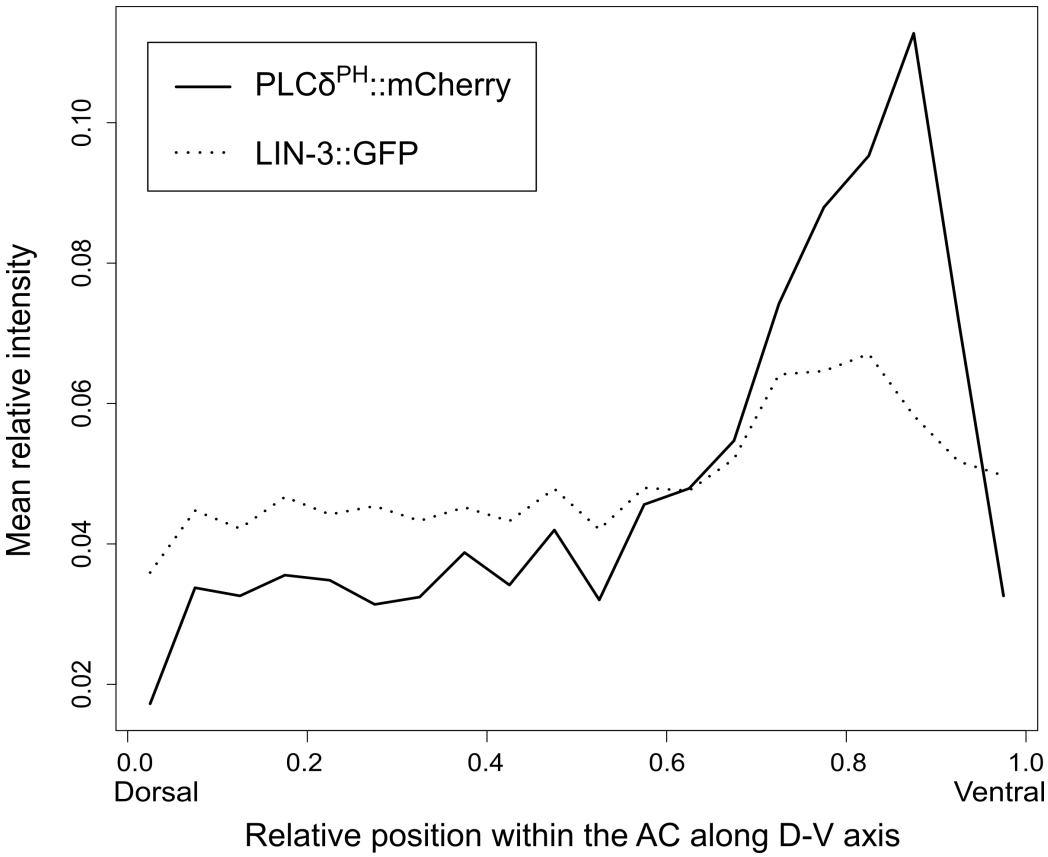


Figure 12: LIN-3 is less polarized in the AC than PIP_2
Polarity profiles of PLCδPH::mCherry and LIN-3::GFP in the AC in wild-type worms at the beginning of the L3 stage. The intensity histogram of the relative fluorescent intensity along the D-V axis of the AC was measured on sum z-projected and thresholded wide-field z-stacks. On the x-axis, dorsal is left and ventral is right. At least 20 animals were analyzed for both proteins.

polarity changes how LIN-3 is distributed along the VPCs and that it is this change that increases or decreases vulval induction depending on the genetic background.

8.3.5 Whole-genome *gap-1* RNAi screen identifies synthetic multivulva genes

We performed a genome-wide synthetic multivulva screen, designed based on the observation that LET-23 mislocalization in a *gap-1* mutant background increases vulval induction [25], using the Ahringer RNAi library [101] and a sensitized *rrf-3(pk1426); gap-1(ga133)* background.

The Ahringer RNAi clones covering chromosomes IV, V, and X, 10'572 in total, were screened for causing a multivulva phenotype in the sensitized *rrf-3; gap-1* genetic background to complete the screen covering chromosomes I, II, and III performed by Peter Gutierrez previously. From these 10'572 clones, almost a quarter, 2'562 did not grow, and for 429, no worms were aliquoted onto the plates. Of the 7'581 RNAi clones that could be scored, 972 (13%) gave rise to a multivulva phenotype in the first round of screening and were subsequently rescreened in the same setting four times. The reason for multiple rounds of rescreening is the low penetrance multivulva phenotype of *rrf-3; gap-1* double mutants. By selecting only the candidates for the ongoing validation that showed the desired multivulva phenotype in several rounds of the rescreen, we hoped to reduce the number of false positives going forward. After the rescreens, we were left with 49 primary candidates that have an synthetic multivulva phenotype in combination with *gap-1* (Tables 1 and 2).

Different mechanisms can be imagined causing such a phenotype, and going on, we had to decide which potential phenotype we want to investigate. As eluded to in the introduction, *gap-1* synthetic multivulva mutation can theoretically be due to any mechanisms controlling LIN-3 distribution. Because we were interested in the particular role of the AC and LIN-3 secretion, we decided to check AC and LIN-3 polarity in a next step. We thought that there might be candidates directly controlling the manner in which LIN-3 is secreted that would ideally leave AC polarity unaltered, but change the distribution of LIN-3. We thus checked AC polarity using a marker for phosphatidylinositol-(4,5)-bisphosphate (PIP_2) and a LIN-3::GFP translational reporter. Four of the 49 primary candidates showed altered LIN-3, but not PIP_2 pattern upon RNAi knock-down. Those were WBGene00044777, *srh-247*, *nlp-26*, and *sra-9*. From these we selected *sra-9*, *srh-247*, and *nlp-26* for further, more detailed validation and analysis.

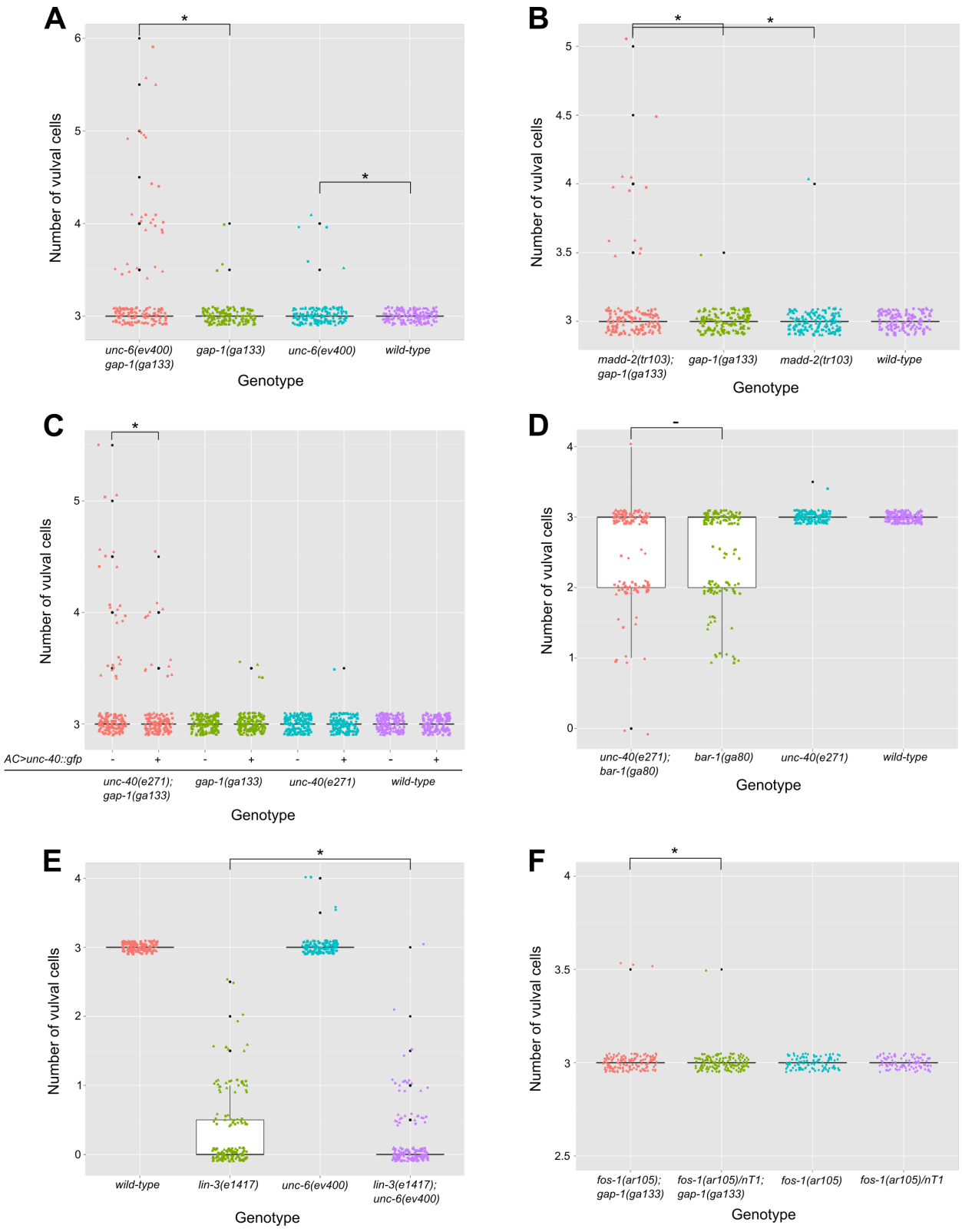

A
Number of vulval cells
unc-6(ev400) gap-1(ga133)
gap-1(ga133)
unc-6(ev400)
wild-type
Genotype
B
Number of vulval cells
madd-2(tr103); gap-1(ga133)
gap-1(ga133)
madd-2(tr103)
wild-type
Genotype
C
Number of vulval cells
AC>unc-40::gfp
unc-40(e271); gap-1(ga133)
gap-1(ga133)
unc-40(e271)
wild-type
Genotype
D
Number of vulval cells
unc-40(e271); bar-1(ga80)
bar-1(ga80)
unc-40(e271)
wild-type
Genotype
E
Number of vulval cells
wild-type
lin-3(e1417)
unc-6(ev400)
lin-3(e1417); unc-6(ev400)
Genotype
F
Number of vulval cells
fos-1(ar105); gap-1(ga133)
fos-1(ar105)/nT1; gap-1(ga133)
fos-1(ar105)
fos-1(ar105)/nT1
Genotype

Figure 13: AC polarity, and not invasion, regulates vulval induction
Scatter and box plots of vulval induction. Black and white are the box plots, the actual data points are scattered in colour. Please note that the scales change from plot to plot. The data for each plot is from evaluating progeny from the same cross. The genotypes are (A) *unc-6(ev400) gap-1(ga133)*, (B) *madd-2(tr103); gap-1(ga133)*, (C) u*nc-40(e271); gap-1(ga133); AC-specific unc-40::gfp*, (D) *unc-40(e271) bar-1(ga80)*, (E) *lin-3(e1417); unc-6(ev400)*, and (F) *fos-1(ar105); gap-1(ga133)*. For every plot except (F), vulval induction was scored in three times 50 animals of each genotype. * indicates a p-Value < 0.05, – a p-Value $>= 0.05$.

8.3.6 LIN-3 polarity is controlled by *sra-9*, *srh-247*, and *nlp-26* independently of AC polarity

The three top candidates from the RNAi screen that showed a phenotype suggesting of controlling LIN-3 polarity (multivulva with *gap-1*, intact AC polarity but changed LIN-3 distribution) were *sra-9*, *srh-247*, and *nlp-26*. They encode two serpentine seven pass transmembrane receptors and a neuropeptide like protein, respectively. There are around 1500 serpentine receptors in *C. elegans*, grouped into diverse classes ([102]). Even though *nlp-26* is classified as a neuropeptide like protein, it has very little in common with any other member of this protein family, and it is predicted to be secreted. It is thought to require processing in order to mature, and one of the genes involved in its processing, *egl-3*, is egg-laying defective, which might indicate a vulval phenotype [103]. Naturally, the fact we found a secreted protein with no clear homologies and two receptors proteins is very suggestive.
Knock-down of *sra-9* increased the number of cells induced slightly, and statistically significant, to 3.05 (Fig.14). Knock-down of *srh-247* resulted in a more confusing outcome. The overall induction dropped to *2.9* cells, a statistically not significant change. Unusually, animals showed both more and less than three cells induced. Even though this change is statistically not significant, biologically, it clearly demonstrates that the reliability of the vulval induction process depends on *srh-247*. Knock-down of all three genes, *sra-9*, *srh-247*, and *nlp-26* markedly disturbed the polarity of LIN-3 in the AC around the time of vulval induction (Fig.15).
These results validate the screen, and even though the scale by which the two tested candidates affect vulval induction is minimal, it is biologically highly relevant.
To confirm the results from the screen, we knocked-out the three candidates

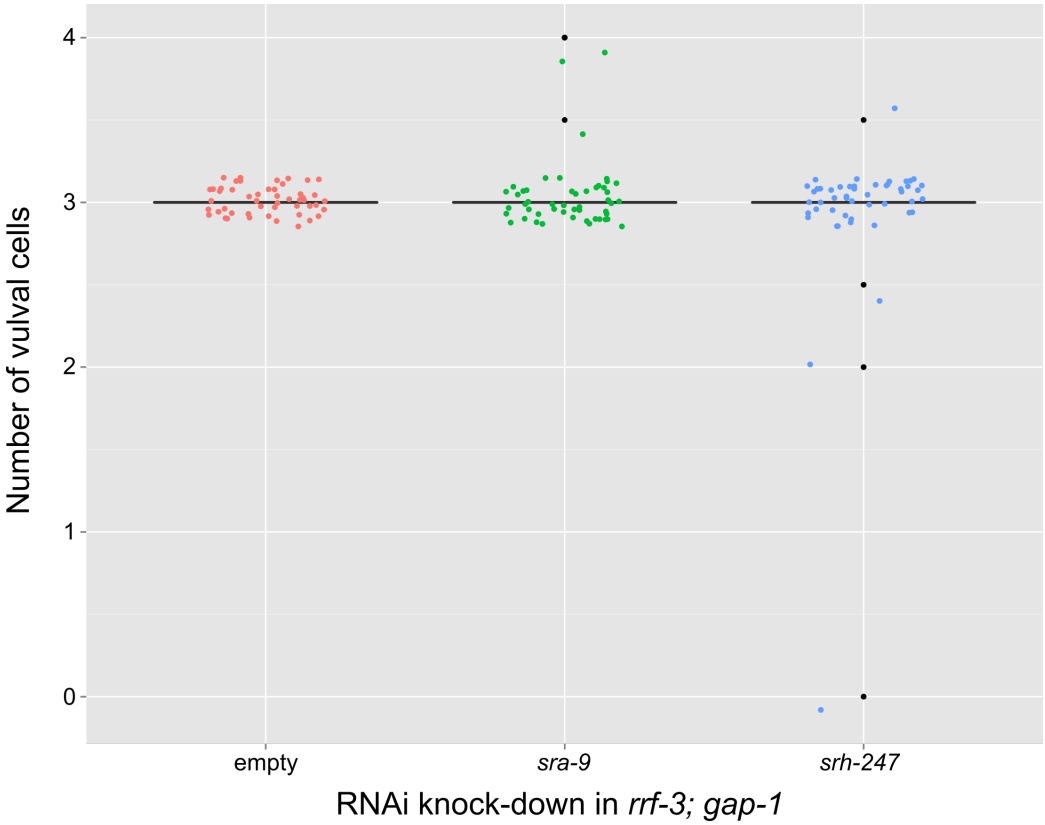


Figure 14: *sra-9* and *srh-247* regulate vulval induction
Scatter and box plot of vulval induction upon RNAi knock-down in an *rrf-3; gap-1* background. Black are the boxplots and the outliers, in colour the individual data points. The 'empty' genotype is the negative RNAi control. Vulval induction was scored in 50 animals each.

using the CRISPR/Cas9 method of genome engineering [7].
(This part of the project is still ongoing, and as mentioned, these results are preliminary. Because of that, this part of the results is incomplete.)

8.3.7 AC polarity is important to center induction on P6.p

Besides the number of induced vulval cells, it is also possible to determine which of the six cell are induced. In wild-type conditions, it is P5.p, P6.p, and P7.p invariably. And even in many mutant with increased induction, P6.p is the cell that adopts the 1° cell fate in the main vulva. We checked this phenotype in AC polarity mutants, but chose only animals with three adjacent VPCs induced. In mutations affecting AC polarity, mainly *unc-6*, 7% of the animals had the induced cells shifted anteriorly, with P5.p adopting

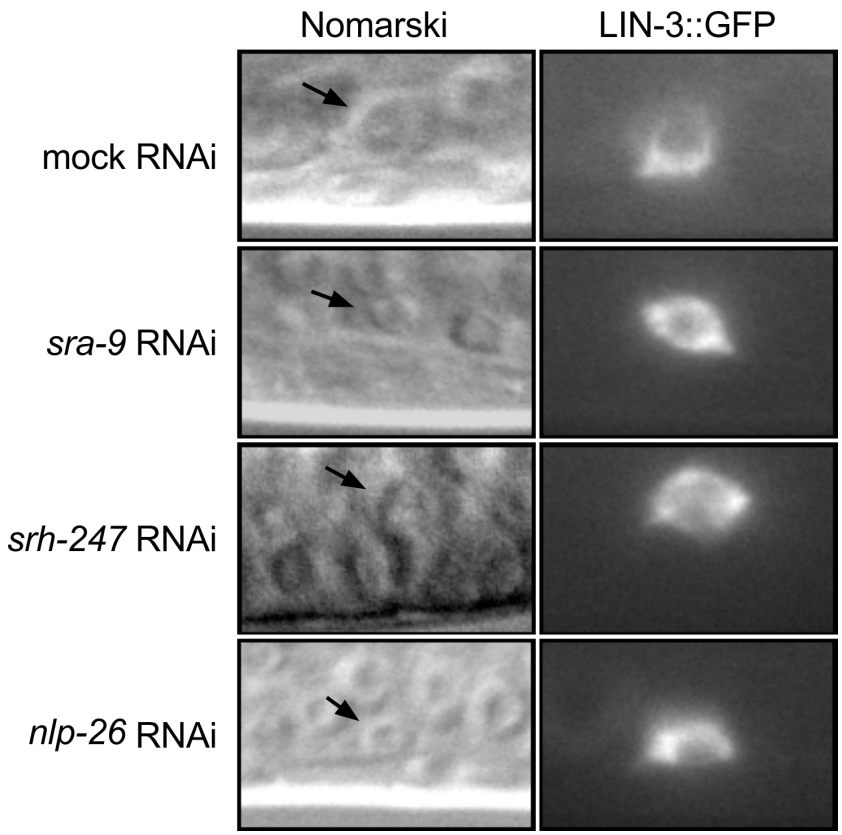


Figure 15: *sra-9*, *srh-247*, and *nlp-26* control LIN-3 polarity in the AC Nomarski and fluorescent microscopy of LIN-3::GFP expressing worms after RNAi knock-down of the indicated genes. Mock marks the negative control. Black arrows point at the AC.

the central, 1° vulval fate (Fig.16). It could be argued that *unc-6* changes AC positioning, and this is most likely part of the explanation, but other mutant backgrounds, including *gap-1*, *unc-40*, *madd-2* and combinations of these had shifted vulval induction, highlighting a role of LIN-3 distribution besides purely ascertaining the proper number of cells being induced.

8.3.8 Discussion

Is AC derived LIN-3 polarized and if so, what role does it play during induction

AC polarity is integral to wild-type development because it is required for forming the connection between uterus and vulva that allows egg-laying and sexual reproduction. The Netrin pathway and its components *unc-6* and *unc-40* are important in establishing polarity, as is *madd-2* and an unknown vulval derived cue [31, 72, 100]. The AC is polarized before invasion however, already during the time of vulval induction at the beginning of the L3 stage. Here we set out to determine whether or not polarity is crucial already at that earlier stage and whether it plays a role in ensuring reliable establishment of the wild-type vulval cell fate pattern.
We found that *unc-6* mutants defective in AC polarity do indeed show a deviation from the wild-type cell fate pattern, but the most common deviation is not the number of cells or a change in pattern, but a change in which VPCs are induced. Only rarely are more grievous effects observed, such as additional induced cells. Similar observations can be made in *unc-40* or *madd-2* mutants, although *unc-6* mutants show the strongest penetrance. Overall the alterations to wild-type pattern are rare, indicating that LIN-3 polarity is not at the core of ensuring proper vulval induction.
gap-1 mutants provide a sensitized background that visualizes LIN-3 reaching VPCs that are not reached in a wild-type background. *gap-1* mutants alone only very rarely have an increased number of cells induced, and if they do, the number of additional cells is very low. *gap-1* mutants thus provide a suitable tool to investigate the distribution of LIN-3. In this sensitized background, the polarity mutants such as *unc-6* show a much more drastic phenotype, in which around 20% of all animals have a multivulva phenotype.This results, compared to the *gap-1* single mutant, illustrates that AC polarity limits induction by most likely controlling the distribution of LIN-3. This finding also highlights the importance of limiting LIN-3 diffusion. Even though *C. elegans* deficient only in AC polarity or *gap-1* are mostly wild-type, a combination of the two results in a synthetic multivulva phenotype. This illustrates the nature and function of redundancy and its importance

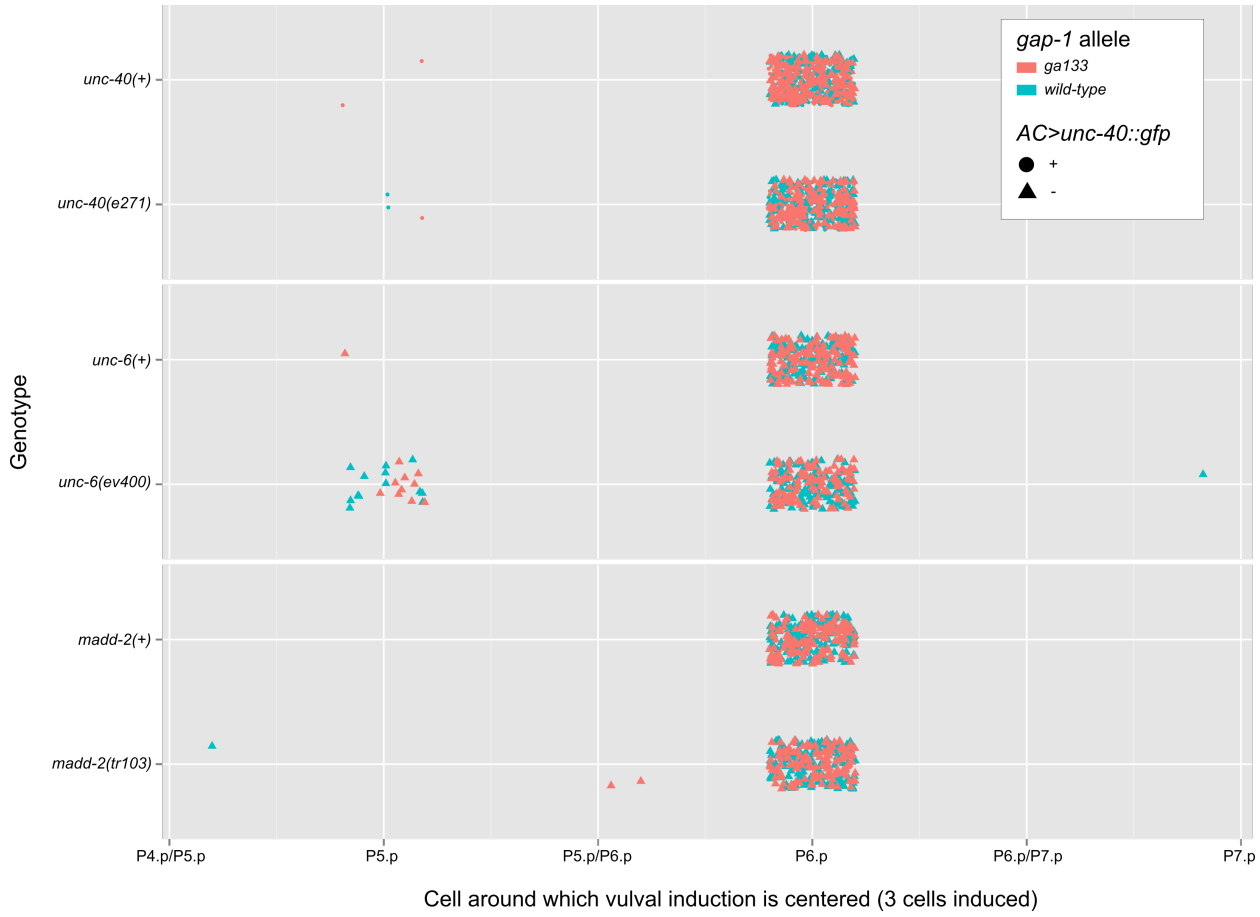


Figure 16: AC polarity is important for preventing a shift in induced cells
Scatter plot of worms with three adjacently induced vulval cells. The genotypes are on the y-axis. Comparisons should be made between the mutant and the corresponding wild-type, derived from the same cross. For *unc-40*, whether or not the animals express an AC-specific UNC-40::GFP is shown by the shape of the marker. The colour is red for *gap-1* mutant or blue for *gap-1* wild-type. The strongest deviation from the wild-type induction pattern can be seen in *unc-6(ev400)* mutants.

for robustness during vulval induction.
The AC is also a highly specialized cell filling a very narrowly defined role during development. It induces one of six cells to adopt the 1° cell fate, while it is separated by two layers of basal laminae from its target. It then breaches through two layers of tissue boundary to establish the connection that allows future egg-laying. And then finally it needs to fuse with a group of other cells in order to make way for the passing of eggs. All this considered, the AC is a highly specialized cell. A cell that has very few, if any, homologues in other organisms. Especially the need to only induce one cell as opposed to induce a group of cells is rather unusual. In higher organisms, cells are much more abundant than in *C. elegans*. As such, the usefulness of the AC as a model for growth factor secretion might be questionable. On the other hand, this *C. elegans* model - like other *C. elegans* models such as learning, chemotaxis, and aging - is necessarily a simplification of any homologous mammalian process. While this might be a point of critique for obvious reasons, it can also be considered a strength. Even though the simplification seems to lose a lot of important components, especially additional cell-cell interactions, the basic principle usually is conserved between homologous systems. In the case of polarized growth-factor secretion studied here, our research shows that such a principle might be important for many processes.
Alzheimers disease and age-related macular degeneration, which causes blindness, have both been correlated with altered secretion profiles [88]. Whether or not changed secretion also causes diseases or is just a symptom is not clear. Surprisingly though, to my knowledge, the developmental role of directed protein secretion is not studied. Generally speaking however, any disease related process also is important during development, and I would be very surprised if directed protein secretion would be an exception.
In fact, the role of the signal producing and secreting cell is the only aspect of morphogen gradient formation and signalling that is largely unexplored. And in the cases where it is studied, findings are limited to genes and mechanisms that permit secretion or do not, as in the case of Wnt signalling and its lipid modifications [104]. This might however be a consequence of the most popular model for studying this and other signalling pathways like *dpp* and *hedgehog*, the *Drosophila* wing imaginal disc. The wing imaginal disc is a mostly two dimensional, epithelial tissue [105] and signalling happening within it is planar. Consequently, the polarity of growth factor secretion would not affect distribution, whereas during *C. elegans* vulval development, the AC is situated away from the VPCs and the polarity of the AC can influence whether the growth factor is released closer or further from its receiving cells. Other aspects of morphogen and growth factor signalling, namely their graded distribution and their reception, have been explored to

various degrees and with different methodologies [106, 107, 108]. Especially the gradient formation has been probed not only biologically but also with mathematical methods [109]. The influence of the morphogen source on its distribution however is often neglected. In this respect, I do firmly believe that the AC as a model for the control of growth factor gradients is immensely valuable. Our results indicate that the polarized distribution of LIN-3 is controlled by a so far uncharacterized pathway that seems affected by overall cell polarity but is independently regulated and maybe even established. If these results can be validated, a new aspects can be added to how cell polarity controls and affects cellular processes.

8.3.9 Materials and Methods

General methods and strains used

Standard methods were used for maintenance of *C. elegans* [1]. *C. elegans* Bristol, variety N2, was used as wild-type reference. Mutations and transgenes used in this study: *zhIs67[lin-3::gfp, unc-119(+)]*, *zhIs98[lin-3::gfp, unc-119(+)]*, LGI: *unc-40(e271)*, LGII: *let-23(sy1)*, *rrf-3(pk1426)*, *qyIs23[$P_c$dh − 3::mCherry::PLCδ^{PH}]*, *ttTi5605*, LGIII: *unc-119(ed3)*, LGIV: *lin-3(e1417)*, LGV: *madd-2(tr103)*, *fos-1(ar105)/nT1[sqIs51, let-XX(m435)] (IV;V)*, LGX: *unc-6(ev400)*, *bar-1(ga80)*, *gap-1(ga133)*, *qyIs66[$P_c$dh−3::unc-40::gfp, myo-2::yfp]*.

Cloning and transgenes

lin-3 was amplified in two part from genomic DNA with primer pairs OMMO118 (tttcctaggCATCGTTGACTGACTCATG) and OJE131 (AGTCGACCTGCAGGCATGCAAGCTgagacacgattctgaaac), and OJE132 (GGCATGGATGAACTATACAAAccttcgtggtttcgtcaa) and OMMO119 (tttcctaggCGACATCAAGGTTCACGG) using Phusion polymerase (NEB). Primers OMMO118 and OMMO119 were modified with an AvrII restriction site. GFP was amplified with primers C (AGCTTGCATGCCTGCAGGTCG) [110] and OMMO77 (TTTGTATAGTTCATCCATGCC) from pPD95.75 (gift from Andrew Fire). These three fragments were fused using fusion PCR and subcloned into pGEM-Teasy (Promega). The *lin-3* fragment was then cloned into pCFJ151 [8] with AvrII.
This was then injected into *unc-119(ed3); ttTi5605* according to protocol [8]. Both extrachromosomal and insertion lines were obtained. The extrachromosomal lines were then used for X-ray based integration.

Microscopy and image analysis

Images were acquired using an Olympus BX61 microscope with an ORCA-ER camera, a Cr.E.S.T. X-light spinning disc, and a Lumencor SPECTRA X light engine, controlled by μManager [111] and Visitron VisiView software. Images were processed using Fiji [112] and Adobe Photoshop. Where indicated, images were processed using Huygens Deconvolution (SVI). Worms were mounted on 3-4% agarose pads and immobilized with 20 mM tetramisole. Images were analyzed using custom written scripts for Fiji and R (http://www.
R-project.org).

RNAi
For the RNAi screen, feeding RNAi was performed as described [101]. Worms were analyzed after 8 days.

Vulval induction counting

To determine the number of induced vulval cells, L4 stage *C. elegans* were mounted and immobilized on agarose pads and observed using a high-magnification microscope. The number of vulval nuclei were counted and used to determine for each half cell whether it divided past the 2-cell stage, indicative of a vulval cell fate, or not. Care was taken to correctly identify every VPC from P3.p to P8.p.

Statistical analysis
Statistical analyses were performed by bootstrapping [113] using a custom written script in R (http://www.R-project.org). Briefly, the acquired data is resampled with replacement, creating many (10000) bootstrap samples. The standard deviation within these samples is an estimation of the standard error of the mean and the mean of these samples an estimation of the mean of the acquired data.

8.4 Future directions and open questions

8.4.1 Was the screen successful?

Peter Gutierrez, a former PhD student in the lab, investigated LET-23 localization during his studies. He did so in two ways, a forward, EMS based, and a reverse, RNAi based, genetic screen. Both screens were based on the finding that the mislocalization of LET-23 results in a multivulva phenotype

when combined with a mutation in *gap-1* that hyperactivates LET-60/Ras signalling. The RNAi screen setup, that we continued as part of this thesis, was very straightforward: A *gap-1* mutant combined with a loss of *rrf-3* in order to enhance RNAi efficiency. This double mutant is then subjected to all the RNAi clones in an RNAi feeding screen on solid media. Initially, only chromosomes I to III were screened. After that, the results did not suggest that the screen worked as intended, as many regulators of AC polarity, as opposed to genes involved in regulating LET-23 and VPC polarity, were found, such as *ina-1*, *unc-40* or *madd-2*. Additionally, control experiments showed that the *gap-1; rrf-3* double mutant has a low penetrance multivulva phenotype on its own. This added another factor of insecurity to the usefulness of the screen. However, later it became clear that also regulators of AC polarity could and should be found and the screen was restarted in order to complete it and to cover chromosomes IV, V, and X in an unmodified fashion, despite the *gap-1; rrf-3* multivulva phenotype. The realisation that also AC polarity regulators could possibly be found meant a great expansion of potential pathways that would make up the screen hits.
The class of candidates we were focussing on initially and that is analysed as part of this thesis is involved in the localization of LIN-3 specifically. But of course, any genes and pathways affecting AC polarity in general should also be found with this screen. This includes the pathways required for AC invasion. So potentially, this screen could also find the missing polarity cue that guides the AC in parallel to *unc-6*. Furthermore, as any multivulva screen, negative regulators of vulval induction will be detected by this screen. This means that this screen is anything but specific, it could find genes that regulate protein localization, polarity and LET-60/Ras signalling. This can be both a strength and a weakness. The strength lies of course in the power to detect genes controlling many different processes with just one screen. This is a weakness at the same time. A follow-up in-depth analysis and re-screen is necessary in order to pick a candidate from the screen to further characterize. Also, because the screen is done in a background that has elevated levels of LET-60/Ras signalling and destabilized vulval cell fate patterns, many false positives can be found. In fact, many multivulva screens have been performed before and any forward genetic screen can be considered practically saturated. It is RNAi that allows systematic knock-out of almost every gene and thus true saturation theoretically. That the forward genetic screens can be considered saturated also means that all the strong regulators have been found and any newly identified gene should be expected to show a mild to weak phenotype only. This is accentuated due to the nature of RNAi and, in case of the LIN-3 localization phenotype, the fact that even strong polarity mutations only give a moderate multivulva phenotype in a

gap-1-less genetic background.
Another caveat of the screen is the use of an RNAi sensitive mutant background, *rrf-3*. The reason to include this mutation is to boost RNAi efficiency, because of the aforementioned problem of RNAi knock-down strength. Since the screen was done in two parts, and the first part did include the *rrf-3* mutation for the reason given, we did continue using the same background. It might have been a possibility to do part two without the RNAi sensitizing mutation to avoid the low penetrance multivulva phenotype. And the low penetrance is relevant, because thousands of RNAi clones are tested on hundred-thousands of worms. Then even a low percentage of spontaneous, RNAi independent multivulva occurrences can become relevant. This constitutes a good reason to switch genetic backgrounds, however we decided not to. One, to preserve continuity, and two, because we were willing to take the trade off between increased power and likelihood of finding relevant genes and higher false discovery rate.
This *gap-1* based screen is certainly powerful and offers the possibility of finding new pathways involved in vulval induction and the prospect of truly saturating these screens. As such, it was the right decision to increase the power of the screen and accept potential false positives as a trade off. It would however be a worthwhile idea to consider adapting this rather broad screen to a more narrow purpose, now that it has been completed. It turned out that most genes found only gave a relatively weak multivulva phenotype. As said though, based on mutant analysis, this is to be expected – especially in an RNAi based screen. There is thus the possibility to enhance the sensitivity of the screen further by possibly including either a weak polarity mutant or to further boost LET-60/Ras to enhance detection of mislocalized LIN-3. Similarly, this screen can be used to investigate other pathways with specific adaptations to the screen set-up to allow detection of all the genes involved. Even if in the near future high-throughput mutant screening should become possible (which is not too unlikely in the light of the recent advances based on CRISPR/Cas9), because even the mutant phenotype of LIN-3 mislocalization in particular is not highly penetrant, a further enhanced sensitized background, not just *gap-1*, should be used.
The question that remains is whether the screen delivered the results we expected. When almost no candidates have been validated and analysed, this is hard to judge. However, there are some candidates that are supporting the notion that the screen delivered what we hoped for. Most notably, both *unc-40* and *madd-2* were found. Both genes are described regulators of AC polarity. Further, a group of genes involved in cell-cycle control was identified. These might be involved either indirectly via control of LIN-12/Notch signalling or otherwise [114]. Other candidates are known regulators of vul-

val induction but not in a way related to localization. Of course, a *gap-1* mutant background does not select for candidates that purely affect localization of either LIN-3/EGF or LET-23, but it increases LET-60/Ras signalling generally. As a consequence, any other gene that does the same would be expected to be found.
Finally, to thoroughly validate this screen, the three candidates we selected need to be analyzed properly. This requires making mutants, reporters, and detailed epistasis. However, at the time of writing, these steps are ongoing, and as such, the final conclusion, whether or not the screen was successful, cannot be drawn.

8.4.2 LIN-3/EGF and AC polarity – is there more?

In my opinion, the idea and the finding that AC polarity influences vulval induction raises many questions worth answering. Not all of these questions could be pursued as part of this project. In fact there are probably still enough open questions to fill another PhD thesis. Here I will mention some of these questions and ideas that I did not pursue during this thesis.
The screen itself potentially offers answers to many different questions, because neither its design nor the multivulva phenotype screened for make too many assumptions.

8.4.3 What happens at the limits of LIN-3/EGF concentration?

A question raised implicitly is what are the limits of the effective LIN-3/EGF concentration range. What is the minimal concentration at which LIN-3/EGF can induce (ectopic) vulval cell fate, and in which relation does this minimal concentration stand to wild-type. It is the LIN-3/EGF concentration that limits vulval induction, because more LIN-3/EGF induces more vulval cells, and controlling LIN-3/EGF thus is crucial. In fact, LIN-3/EGF signalling during induction is buffered and even tripling the dosage of LIN-3/EGF does not change the number of cells induced [63]. We identified AC polarity as an additional factor that helps ensuring wild-type vulval induction. However, how crucial its role is is not fully clarified yet. To test AC polarity and its function during induction more thoroughly, combining AC polarity mutants with increased LIN-3/EGF will be necessary. Such experiments could more clearly highlight AC and LIN-3/EGF polarity as part of the vulval induction buffering mechanisms.
A complementary approach to test the limits of vulval induction is to focus on LET-23 sensitivity. Even if LET-23 is mislocalized, it signals strongly enough to induce vulval cell fate together with a *gap-1* mutation. AC depolariza-

tion lowers LIN-3/EGF concentration and a lower LIN-3/EGF concentration reduces the number of signalling LET-23 molecules. A combination of AC depolarization with LET-23 mislocalization and a *gap-1* loss-of-function would potentially highlight how sensitive the inductive process really is. This would be interesting because vulval induction should not happen spontaneously and always require a signal. So even if LET-23 should be randomly activated and signal, this must not result in the acquisition of a vulval cell fate. How and whether there is a regulatory mechanism preventing this could potentially be tested by the proposed experimental set-up.

8.4.4 Is it really LIN-3/EGF?

A key question I was not able to address during my PhD studies is whether there are molecules besides LIN-3/EGF that contribute to increased vulval induction in *gap-1* mutants with depolarized ACs. Another way to ask this question is whether there are other ways that AC polarity affects induction other than via LIN-3/EGF polarity. The answer in a simple experiment: Compare vulval induction between AC polarity mutants with and without LIN-3/EGF. However, the setup is not trivial due to the nature of *lin-3* mutants. A *lin-3* knock-out is not just completely vulvaless but also lethal. So even if the lethality would be rescued, without any vulval cells induced, the strength of vulval induction cannot be compared. Additionally, a good experimental setup should allow potential induction change to go both ways, more and less cells. A genetic background suitable could be the *let-23(sa62)* gain-of-function allele. Because this is a weak gain-of-function, it might have to be combined with a *gap-1* mutation. Like this, vulval induction would be independent of *lin-3*. The other problem is to completely remove LIN-3/EGF. It is a problem because *lin-3* null mutants are lethal. Alternatively, LIN-3/EGF could be removed by eliminating the AC by laser ablating the somatic gonad precursor cells. But because the question is what influence AC polarity has, the AC is required. A true alternative might be to combine *lin-3* loss- and reduction-of-function alleles. This approach is neither particularly clean nor very promising, because even the strong loss-of-function *lin-3(e1417)* allele was not strong enough to do this particular experiment. A more laborious, but potentially more promising approach is to knock-out *lin-3* tissue-specifically. This could be possible using an FRT-based genome-engineering approach. With the right promoters, somatic *lin-3* null mutants could be generated in the vulva only, without affecting viability or the AC itself.

8.4.5 What is the significance of the centration phenotype?

One of the phenotypes we found AC polarity affected is which vulval cells get induced. AC polarity mutants have a clear tendency to induce cells other than the wild-type P5.p, P6.p, and P7.p. This phenotype is not widely acknowledged. In wild-type, the induced cells are always the same. Why this is so is not known, and whether a shift in vulval induction affects fertility or mating is not known either. It thus seems to be a minor phenotype with little to no impact on development, and yet there is no variability. It is in fact just as fixed as the number of cells induced in a wild-type situation. The discrepancy between apparent lack of biological importance and fixed phenotype I think is interesting and a good reason to investigate the developmental consequences of a shift in vulval induction.

8.4.6 The other candidates from the screen

As mentioned, the candidates from the screen are likely regulating more than AC polarity and vulval induction. Originally, it was designed to identify genes localizing LET-23 to the basolateral side of the VPCs. But candidates could very likely regulate other processes too. For example, regulators of AC invasion could have been identified because AC invasion is tightly coupled to AC polarity. Many other processes could regulate vulval induction, even some we do not know yet. And this is the truly interesting perspective. An in depth look at the 50 candidates thus could offer new insights into vulval induction, the early role of the AC, and maybe even shed light on the complex interplay between the AC and the vulval tissue.

8.4.7 Other related projects

Textbook models of most, if not every, signalling pathway cover primarily the genes and molecules related to signal reception. Little is known or little attention is paid to the signal, how it is produced and how it is sent. What we describe here is the role of the signal sending cell. And it illustrates that even if it is not the major factor in modulating signalling, it contributes to the outcome of signalling. Our results illustrate that in cell-cell signalling, both the sending and receiving cell play an important role in controlling the signal. And I think that the signal sending cell has been a mostly neglected aspect of cell-cell communication.
Signalling is rarely linear. It is amplified, dampened, or integrated with other pathways. The focus of trying to understand how this happens lies on the processes in the signal receiving cell and includes how the signal is received, how it is transduced and what response it elicits. We showed that the signal

sending cell controls which cells the signal reaches at which concentrations. And obviously,the presence of the signal is controlled by the sending cell too. Potentially, there are even more layers of control and signal modulation. The signal sending cell could modify the signalling molecule, changing its half-life, receptor affinity and the ability to signal itself. The signalling cascade does not start with the receptor, but with the signal. And the signal can be modulated, not just the response.
I think that vulval induction as a model is well suited to further study how the signal sending cell influences signalling. I outline here a few possible experiments and questions.

How is LIN-3/EGF processed?

LIN-3/EGF is synthesized as a transmembrane protein. The EGF domain that signals needs to be processed and cut from the membrane spanning domain to diffuse, but necessarily to signal [115]. In *C. elegans*, the protease responsible is not known. *rom-1* is the homologue of the Drosophila protease that cleaves LIN-3/EGF, but in *C. elegans*, it cleaves LIN-3/EGF only in the VPCs [67]. Because the protease has not yet been identified in a vulvaless screen suggests one of two things: A LIN-3/EGF protease null mutant is lethal, or the protease is dispensable for wild-type vulval induction. In the latter case, our findings might offer a novel approach to finding the protease. We describe that a broadened LIN-3/EGF gradient increases vulval induction. This implies that LIN-3/EGF is released from the AC and diffuses in order to form a gradient, which would depend on the protease. Consequently, a loss of the protease would limit the spread of LIN-3/EGF. The *unc-6 gap-1* double mutant has increased vulval induction because LIN-3/EGF forms a broader gradient than normal, we hypothesize. A loss of LIN-3/EGF protease in this genetic background should theoretically suppress the multivulva phenotype. And to screen for suppression of a stable, even low penetrance multivulva phenotype is doable. Such a screen would also identify other factors that facilitate LIN-3/EGF diffusion. Ideally, such a suppressor screen would be carried out using a mutagen such as EMS. However, this poses a problem because any other loss-of-function mutation suppressing vulval induction, first and foremost a *lin-3* mutation, would be identified too. This is a general problem studying the *C. elegans* vulva. The model is saturated with random mutagenesis screens and any forward genetic screen is unlikely to find anything new. The alternative approach is to do a reverse genetic, genome-wide RNAi screen, where every gene is knocked-down individually. A targeted RNAi screen based on in silico analysis would reduce the amount

of work. Such an approach relies on the quality of the selection and accurate gene predictions. It is also unlikely to identify novel components of LIN-3/EGF processing. Other screening methods might include cell- or tissue-specific mutagenesis using an error-prone DNA polymerase, selective increase of DNA replication errors by tissue-specific RNAi knock-down of the DNA repair mechanism, or a combination of both. To my knowledge, such screening strategies have not been successfully tried before however. But these potential approaches might avoid the problem of having to filter out all the VPC specific mutations in the LET-60/Ras pathway that are already known to restrict vulval induction.
Besides uncovering novel regulators of LIN-3/EGF secretion, identifying modifying enzymes that change diffusion and signalling properties of LIN-3/EGF would be very interesting. Another question is whether and how LIN-3/EGF diffusion is affected by its substrate, like the basal lamina or the extracellular matrix.
In conclusion, during cell-cell communication, the role of the signal sending cell is poorly understood. How production and release of the signal is regulated, whether the signalling molecule is modified and what factors control its diffusion are all open questions. And until we understand the signal sending side of cell-cell communication, an important regulatory mechanisms remains ignored.

8.5 Conclusions

This projects consisted of performing the second half of a genome-wide RNAi screen that was originally designed to find genes controlling LET-23 localization. However, it was reframed as also looking for genes affecting LIN-3/EGF distribution generally. The new idea that motivated us to complete the screen in the first place was that the LIN-3/EGF producing AC might be involved in controlling distribution too. In a wild-type situation, LIN-3/EGF is polarized towards the VPCs, which is ensuring reliable induction. In combination with elevated LET-60/Ras signalling, AC and LIN-3/EGF depolarization can change the wild-type inductive pattern, which we demonstrated using *gap-1* and AC polarity mutants. Vulval induction in a vulvaless background also decreases even further upon AC depolarization. On its own, AC depolarization is sufficient to shift the induced cells. AC and LIN-3/EGF polarity is thus not crucial for wild-type induction, but it is seemingly one of many mechanisms that contribute to reliable vulval induction, even when there are fluctuations in signalling during induction. The completed RNAi screen resulted in 50 candidates, of which we selected three for further analysis due to their phenotype pointing towards a specific role in LIN-3/EGF

polarity. The three genes are two serpentine 7-pass-transmembrane receptors, *sra-9* and *srh-247*, and one secreted, neuropeptide-like protein lacking any homology to other neuropeptides, *nlp-26*. Their phenotype is characterized by altered LIN-3/EGF distribution but unchanged AC polarity upon knock-down, suggesting that the LIN-3/EGF distribution is controlled separately from general AC polarity, but not independently from AC polarity. Whether this is special for LIN-3/EGF or a general characteristic of growth factors is unknown, and what the benefits of such a polarity system within a polarity system are is hard to tell. It might ensure proper control of subcellular distribution by having a dedicated mechanism or be necessary in context of the AC, a cell that lacks the standard epithelial polarity features. It might be interesting though to try and modify *lin-3* to be transported by the standard apical trafficking system and determine in which way that changes vulval induction.
If anything, I hope we could highlight the role of the signal sending cell in cell-cell communication, and that it is not just that of producing the signal and then leaving everything up to diffusion and the receiving cell. Instead, the producing cell, at least during *C. elegans* vulval induction, actively controls the distribution of the signal LIN-3/EGF, and possibly other aspects of signalling too.

9 Discussion - Part 1

9.1 *Caenorhabidtis elegans* research

9.1.1 What could be the next step in *C. elegans* research?

Sydney Brenner originally stated that he picked *C. elegans* as a model to study neurobiology because of its simplicity. And whilst in my opinion trying to understand learning using such a simple organism would probably not allow too many conclusions regarding how we learn and memorise, the basic function and set-up of neuronal networks in general can certainly be studied very well, as was recently demonstrated very nicely. But *C. elegans* showed to be an extremely potent genetic tool to do much more than just neurobiology, revealed a great many biological principles and allowed the in-depth analysis of many signalling pathways. The trend for the future seems to be in the direction of high-throughput analysis, analysing whole processes and systems at once, like whole cell transcriptome analysis. What this approach does not cover though is the intricate details of the interactions of individual molecules. This is something that *C. elegans* might be suitable for because of its relative low complexity and reduced redundancy. Many pathways could thus be

studied with less effort compared to higher organisms. On the other hand, the time might now come that it will be possible to put all the individual genes together into pathways and start to find out not just what they do on their own, but how they interact with others.

9.1.2 What and how much can *C. elegans* still teach us?

And when, if ever, do we need to change models? *C. elegans* is a great model because of it is minimalistic and simple. This is great to study processes that are not dependent on size of the organism. So anything that happens on a cellular or tissue level can translate with relative ease to higher organisms. However, as soon as it is about understanding organism-wide processes, simple life forms such as *C. elegans* are suboptimal when in the end we want to understand how we work. On the other hand, we are still far away from understanding how even pathways interact. And for something like that, *C. elegans* is nicely suited. Also if we eventually want to understand how complex organisms function, we should first try to understand the workings of simpler organisms. Once again, *C. elegans* is very well suited to try an undertaking like that, because basically it is digestive and reproductive tract, with some neurons on the side.

9.2 The Anchor Cell

At the beginning of this thesis was the AC, and the then freshly hyped research area of AC invasion. And the first half of this thesis was spent on putting *madd-2*, a gene that was identified as a potential LIN-12/Notch target involved in vulval induction, into the framework of AC invasion regulators. Towards the end of this first half, several pieces, some old, some new, came together and revealed a potentially new and exciting role for the AC at the beginning of vulval development, during induction. This occupied me for the latter half of my thesis and it hopefully constitutes the beginning of a fresh way of looking at the AC and its role during induction.

9.2.1 Why do we study the AC?

The obvious answer is to understand metastatic behaviour of cancers. This is what popularized studying the AC in the first place. I do however think that there is more to this cell than just invasion. During *C. elegans* development, invasion is but one job that the AC has. It in fact needs to coordinate the development of the two organs most tightly involved in reproduction, the uterus and the vulva, and as such they are very crucial to evolutionary

success. I mentioned the switching of roles the AC has to perform, and I think that it is worth trying to understand this highly complex task. This might offer new insights into the coordination of developmental events and their regulation.

9.2.2 How will we progress in understanding the AC?

With the advent of genome editing, it might become possible to characterize the AC in more high-throughput manners. This might in turn offer new insight into the transcriptional networks regulating invasion and which genes are important to carry out invasion. I also believe that, especially based on the results from the second part of this thesis, the process of vulval induction and the role of the AC therein should be re-examined. Because I believe that the way we look at this example of cell-cell communication adds complexity to the role of the signal sending cell, which was largely ignored so far and thus this phenomenon might be more common than previously thought.

9.2.3 What is missing to understand the AC?

I think, what this thesis illustrates is that the answer to the question what is missing in understanding the AC or any other process is heavily dependent on our ability to ask new questions. Besides that, I do think there are still some obvious gaps in our understanding. From the beginning, it is not clear how the cells of the somatic gonad divide properly in a way that allows the two AC/VU precursors to be positioned as such that they can engage in reciprocal LIN-12/Notch signalling. It then further is not understood what exactly determines the outcome of the AC/VU decision. Then follows an apparently more intricate dialog with the vulval epithelium including induction, invasion and morphogenesis, which most definitely still is not understood properly. The most obvious example of this is the still missing secondary cue that guides AC invasion.

9.3 Invasion and Metastasis formation

9.3.1 Trying to understand metastasis with model systems

In research, the model system closest to us humans can be argued is human cell lines, because they have the same genetic make-up and share many traits found in our cells. However, cell lines are isolated cells and thus cannot represent the complexity that is an intact organism. Nevertheless, these cell lines are a good tool to investigate different aspect of the biology of our cells and to a certain degree of our organism. Most of these cell lines are in fact

derived from cancer cells, because they have all the characteristics that enable them to proliferate in an artificial setting *in vitro*. One could argue that this is helpful in trying to understand and eventually cure cancer. However, as mentioned before, no two cancers are alike. With respect to metastasis again, cancer is not cancer. Also, not every cancer forms metastases and not every cancer cell does either. In fact, it is entirely possibly that even cancer metastases can no longer form metastases themselves, but have lost this specific trait. In conclusion, just studying cancer cells to understand metastasis, because of their diversity, or genomic instability, and because of the potential flexibility with respect to their gene expression profiles, might prove not successful.

The huge diversity among cancers complicates studying any cancer trait in cancer cells. As alternative, the study of the physiological equivalents could provide the basic insight to start and understand what happens in a cancerous situation. And ideally, these studies will be done in different organisms and different models in these organisms in order to explore all the different mechanisms that enable cells to invade. Of course, metastasis formation is more than just invasion. It requires cell to leave an environment, remain viable, invade at least twice, in and out from the bloodstream, and finally to adapt to the new environment they encounter, which might possibly be the biggest hurdle. However, as different cancers derive from different organs and form metastases in different organs, too, the common denominator that metastasising cancers share is the invasion process. As such, this step could be the one that enables a breakthrough in controlling cancer metastasis formation, because it is not just common to all cancers but also should be a similar process in every cancer, because of the conserved nature of tissue borders. Cancer cells are adaptable and varied, this makes them so difficult to combat. But by trying to understand the core machinery, upon which also these cells rely, a beginning can be made. But even so, because they are so very versatile and can adapt, cancerogenesis will enable these cells to evade and find detours, potentially avoiding even core components and mechanics of cell invasion. Thus, the question whether we will ever be able to reliably model cancer cell metastasis is without a straight answer. Be that as it may, our knowledge is still limited, and the strength of basic research is that it does not limit itself to pursuing foreseeable topics, but to be open for new, exciting discoveries that can potentially change the way we deal with not just cancer, but cell biology and medicine overall.

9.3.2 AC invasion as a model for metastasis formation

A big reason why the topic of AC invasion became so popular is that it shares components with what happens during metastasis of cancer cells. The best demonstration of this comes from an experiment where the ability of invading cancer cells to penetrate chicken allantoid membrane was tested following the knock-down of components of AC invasion [21]. Indeed, some components that are helping the AC breach the basal laminae during invasion do the same in invasive cancer cells in an quasi *in vivo* setting. Despite being not purely *in vitro*, this experimental set-up still differs markedly from true *in situ* and only approximates the *in vivo* setting. For example, cancer associated stromal cells are missing, which are most likely crucial to cancerogenesis. Also, this specific experiment used cancer cell lines, as opposed to primary cell cultures. The problem is that all cancer cells are different and very quick to adapt to new environments, it is one of the traits that is selected for during metastasis. Because of this, any cell lines most likely do not represent any actual cancer cells precisely. Whilst it is still a good approximation, it only is an approximation, and that should be considered by interpreting any result derived from such an experiment. However, as researcher, we depend on money from different sources, which usually include funds dedicated to cancer research, and as such we tend to stress what the similarities are and to forget in which way the model might not be as close to the in vivo situation as we would like it to be. Thus, I question really how good of a validation it is, if cancer cell lines in a quasi *in vivo* setting behave similarly to AC invasion. Instead we should try and understand AC invasion as a model for cell invasion generally. I mentioned previously that I think more emphasize should be put on understanding the physiological cell invasion process, and not primarily metastasis itself. The reason for that is that metastasis happens in conditions that vary wildly which makes it difficult to find the exact components involved, while physiological or developmental cell invasion happens reproducibly, thus enabling systematic analysis. And then the next step should be to find the link from an understood cell invasion program to metastasis. AC invasion is certainly well suited to study developmental cell invasion, but I do think that other models too should be used, so that a common and conserved core cell invasion program can be discovered.

9.3.3 Does knowledge of *madd-2* contribute to understanding metastasis?

Even though this thesis and the research I did is clearly basic, ideally we would like to contribute towards medical understanding and eventually to

healthcare improvements in one way or another. *madd-2* is a good candidate for this because it is the homologue of the human disease gene *Mid1*. Opitz syndrome caused by *Mid1* mutations is characterized by midline closure defects [79]. There is no direct implication in cancerogenesis so far, only a correlation with increased invasive behaviour in a comparative transcriptomics study [116]. What we described during our work on *madd-2* can be summarized as trying to understand how *madd-2* interacts with the UNC-6/Netrin pathway and AC invasion. So we looked at a *C. elegans* phenotype of *madd-2/Mid1* for which there is no clear equivalent in humans. However, we could highlight the novel mechanism of indiscriminate invasive behaviour which is suppressed by *madd-2*. Even though this does not add directly to the understanding of Opitz syndrome, because the model we studied is not midline migration but cell invasion, or the understanding of metastasis, because *Mid1* has not been shown directly to be involved, we do describe a mechanism that might help understand both Opitz syndrome and metastasis. Interestingly, just like the dual phenotype of *madd-2* which both enhances and suppresses AC invasion, Opitz syndrome and metastasis are characterized by both too few and too much migration, respectively. Potentially, a single mechanism, like indiscriminate migratory/invasive behaviour that does not respect guidance signal, might be contributing to both human diseases.

9.4 The signal in cell-cell signalling

9.4.1 Cell-cell signalling and the signal sending cell during vulval induction

The vulva of *C. elegans* is a well established and potent model to investigate various aspects of cell-cell signalling and how various pathways interact. Especially the induced cells, the VPCs and then the vulval cells, have been characterized to a great extend with regards to the competence and especially the inductive process. However, the other important component of vulval induction is often considered a mere signal production site and nothing more, the AC. The fact that the AC has recently spiked interested for invasion did not add to the notion that what happens during induction should be better understood and instead focussed the effort on its later roles during development. Hardly anything is known about how the signal is produced, except for a transcription factor, *hlh-2*, that is involved in the process. Other than that, the AC is just the cell that produces LIN-3/EGF and that supposedly secretes it in the shape of a gradient. Essentially, this is how much is known about the cell that sends the signal initiating cell-cell communication. The amount of knowledge is immensely skewed towards the understanding of the

receiving aspect of cell-cell communication. But without a signal that initiates the process, without another part besides the signal receiving cells, there would be no communication and no vulval induction.

9.4.2 The ligand in cell-cell signalling

Besides the signal sender and the signal receiver, the third component in cell-cell communication is the signal itself. In the common description of signalling pathways, the signal is acknowledged, but treated merely as a trigger for the complex intracellular signalling cascade. However, the signal, typically the ligand for the receptor atop the cascade, is crucial for setting off the cascade. And not only does it need to be present in the first place, it needs to be present in sufficient quantities and in the right place to meet the receptor. To assure this happening, the distributions of both receptor and ligand have to be controlled. But more often than not, we assume a ubiquitous ligand distribution and only treat the receptor as the one component whose distribution can be controlled. Because it is arguably easier for the signal receiving cell to control how the receptor is distributed, it is integral to the cell membrane in most cases. The ligand however usually is dispatched from the signal sending cell and moves randomly once it is released. But just because we assume the movement to be dictated primarily by the laws of physics, could it not in fact be easily imagined to be steered and controlled in a more strict fashion? For example, adaptor molecules can and do change its diffusion behaviour, the composition of any extracellular matrix can be and is attractive or repulsive towards the ligand, and other cells could even produce ligand degrading enzymes to prevent it from spreading [109]. All this is relatively simple and there is no good reason why there would not even exist much more complex and more subtle modes for controlling ligand distribution. We do not know the role of the ligand in this process. Potential ligand modification is not unreasonable to assume, and such modifications could change the affinity towards the receptor, the ability of the receptor to activate the cascade, or even recruitment of co-receptors to change signalling. In fact, some modifications of Wnt have been observed that do not only change its diffusion behaviour, but also its signalling [104]. More research is needed on this topic though to generate a more complete understanding.
Biological systems are usually highly complex, including many different regulatory mechanisms. We focussed so far on the ones that are a part of what we perceive as the signalling cascade, the core of any pathway. We limit the potential complexity to the signal receiving cell, and see the ligand simply as a start button that can either be pressed or not be pressed. I think, it would

be weird if it was that simple, however. Biology tends to take the apparent complex and cumbersome route to solving problems. So thinking that the key part in an essential mechanism such as cell-cell signalling should be so devoid of any meaningful way to manipulate it strikes me as odd.

9.4.3 Vulval induction compared to other models for cell-cell signalling

Ultimately, despite many similarities on microscopic and molecular levels, a worm is far from a human. The starkest difference lies in the number of cells, roughly 1000 cells versus more than one billion times this amount [117]. Further, *C. elegans* is mostly a digestive and reproductive tract, held together by some muscles and a basic nervous system, whilst humans have many specialized organs and an immensely complex neurological make-up. The contrast seems so enormous that it could be believed that nothing is conserved, but in fact *C. elegans* is very useful to understand certain aspects and basic principles of how humans, and any other organism, function. Of course, as iterated many times in this thesis, the similarities come from the microscopic and molecular level, it is the cells and genes and proteins that make human and worm more similar than would be imagined. However, the macroscopic differences should not be forgotten, and they are most definitely huge, in more than one sense. Arguably, for some models and processes, this difference in cell number might be almost negligible, however, not for cell-cell communication, I would argue. Naturally, the basic premise remains, signal production, signal reception, response. And maybe the response and reception part, which has mostly been the focus when looking at cell-cell communication, might even be similar. However, where the signal comes from in higher organisms, including humans, is most certainly not one individual cell. Even though the principle is the same, practically I think this makes a considerable difference, especially considering what I was working on during this thesis.
We showed as part of this thesis that a precise control of growth factor distribution can be crucial to ascertain the desired response from the signal receiving cells. But we did so in a situation with one cell that produces the signal and six cells that are competent to receive it. Furthermore, the cell sending the signal, the AC, sits just next to the receiving cells, separated only by two layers of basal lamina. Because the source of the signal is a single cell, the shape in which the signal is released and distributed can be tightly controlled. This is necessary because it also matters how many cells exactly receive the signal. Whether it is three or four or even only two actually matters a lot in *C. elegans*, making the difference between wild-type,

multivulva, and vulvaless phenotypes. In humans, with so many more cells, and a cell lineage that is not fixed, this absolute precision in how a signal is distributed and where it reaches exactly is less crucial, as one cell more or less receiving or not receiving a signal is in all likelihood not important. Because of this, it is questionable whether or not the research done as part of this thesis, and the principle of growth factor distribution being controlled by the signal sending cells has anything equivalent in higher organisms, including humans. Such highly detailed control might be unnecessary on a larger scale, however, we do not know. Thus, if anything, the research we did should highlight that there is more. That there is a potential layer of control that does not act within the signal receiving cells. And this additional control could be of interest and offer another way in which biological systems can be shaped. It is very likely not the case, as I explained, but we will not know unless we check.

9.4.4 Why is cell-cell signalling so focussed on the signal receiving cell

As is the case with neglecting the signal sending AC during vulval induction, in other models too is the focus heavily on the cells that receive the signal. There does not seem to be a good reason for this, but there might be an obvious one. In the signal receiving cell, a whole cascade of signalling molecules spring into action upon receiving the go-ahead in form of the sent signal. In terms of research, there is many a gene to investigate and many gene-gene interactions to characterize. On the other hand, in the signal sending cell, there seems just to be the signal, and when this is missing, nothing is happening in the receiving cells. Arguably, this also makes it more difficult to study. If the signal is lacking for any reason, because the signal itself is mutated or any component in the pathway leading to its production, the phenotype in the signal receiving cells, and these are the cells that are usually easy to observe, is the same as when the receptor, the initiator of the signalling cascade, is missing. The task to study what is happening in the signal sending cell is thus both difficult and unattractive, and so is the question how the signal itself contributes to cell-cell communication in other ways than just initiating the cascade in the receiving cells. But even if these reason might be valid, we are essentially trying to understand an equation by looking at just one half of it. Cell-cell communication is we think largely made up of complex signalling networks in the cells receiving the signal, but maybe we just picture it that way because we do not imagine what is involved in regulation of the production of the signal and what is needed to successfully deliver the signal to its targets. I think it is no longer acceptable to just put

the signalling molecule in a pathway when describing it, and that we need to start understanding how the signal is produced, how it might be modified, how it modifies signalling, and how it reaches its receptor. The next step in understanding cell-cell communication should be the understanding of the other cell, to form a whole picture.

9.4.5 What does vulval induction as model for cell-cell signalling add to the understanding

Cell-cell communication is a ubiquitous process and most models studied offer some insight into how cell-cell communication works. So why do we study this process in a nematode? The advantage of the vulva of *C. elegans* as a model system is that signal strength and efficiency can be closely monitored. The number of VPCs is clearly defined and how many receive sufficient signal is easily found. Like that, quantification and epistasis analysis is rather simple and allows for in depth investigation of different signalling pathways and how they interact with each other. Furthermore, the signal, LIN-3/EGF, is produced by one single cell for the purpose of vulval induction, which again clearly defines and limits the system, making it easier to track and factor in the different variables into how they might contribute to the final outcome, which is how many of the six cells are induced. In many ways thus, vulval induction as a model to understand cell-cell signalling and communication is ideal because of its simplicity. This however might also be considered a downside, it is after all the complexity of higher multicellular organisms which we want to eventually understand. Nevertheless, the study of *C. elegans* vulval induction lays a solid foundation in the form of understanding the signalling pathways, how they interact and how cell-cell communication works in its simplest form, upon which the study of more complex, bigger systems can build.

10 Discussion – part 2

In the following section I would like to shift focus. Above, I discussed the results of the research I carried out during my PhD. In the following, I would like to not discuss results, but research itself. This might seem unorthodox and maybe even out of place, after all, this thesis is about biology. However, research, and especially the way research is done, is changing rapidly these days. And even though I will not discuss how our scientific apparatus is organised and functions these days, I would like to discuss how science is done, and what I see as the crucial aspects of the scientific process.

10.1 The scientific process and the importance of asking questions

The fields of developmental and molecular biology, genetics, cell biology have a well-defined toolset, which is so potent that most any question can be answered. That said, the more complex the question that we are asking is, the more complex the answer tends to be. Sometimes the answer can be complex that it is difficult to fully comprehend. Finding the answer is possible in most cases nonetheless. It follows that the most difficult task any biologist has today is to be able to ask the right question. To ask the question that gives an answer that is understandable and that advances our knowledge. It is this, and not the ability to get an answer, which is the challenge.

Science is driven by curiosity. And our curiosity has been very successful at pushing us to find answers to many different questions and creating a huge body of knowledge. In fact it sometimes seems that our initial curiosity is satisfied by what we already know. The difficult task is thus to rekindle the curiosity at the stage where there are no more answers and where we have to find the answers ourselves. This is the hard part. The source of curiosity is the absence of knowledge. But to get to the point at which there currently are no more answers, many answers have been given along the way, and we experienced all the knowledge that we have gathered.

Another difficulty lies in telling apart true knowledge, based on facts gained from experiments, and experience, which predicts and extrapolates what will happen, without experimental confirmation and observational findings. I make the difference between true knowledge and experience because the step from knowing what happens to knowing why it happens is where knowledge is gained.

It is this step, asking the question 'why?', which is the crucial one. It sounds trivial, and once the question has been asked it seems trivial too. In my experience, it is anything but. The reason for that is that it is hard to tell apart true knowledge from experience. To give an example, at the end of vulval development, in the fourth larval stage of *C. elegans* development, the vulva has reached a stage called 'X-mas tree', called so due to the shape of the lumen. But in the adult, the vulval lumen has collapsed. It is experience that tells us this is how it happens. And the textbooks refer to this process as eversion. But the question how and why eversion happens at that stage, what is required on a molecular, and what on a microscopic level, is not known. The answer would be rather easy to find, if only the question would be asked. The difficulty is to recognise that we know from experience that eversion happens, but that we do not know how it happens. It is difficult

to be curious still, in the light of all that we already know about vulval development. It is difficult to go on and continue asking 'why?' when dealing with the most obvious seeming processes.
In my view, it is not the success of finding an answer, but the ability to ask the question which makes a good researcher.

10.2 Textbook models and paradigms

Paradigms are encountered frequently in molecular biology. They are very useful to get a general understanding of all the complex mechanisms at work in and between our cells. But because everything is so complex, and almost anything that can possibly happen does happen in some context, they are usually also at least partially wrong. Paradigms can thus be dangerous, especially when switching from training and learning to researching. They are dangerous because they limit us. They limit what we would consider when designing experiments and when we try to ask questions. They erect borders and set rules in the absence of both. Borders and rules do make things easier, as do paradigms. Simplifying can be helpful when trying to make sense of molecular biology, but it can also create lots of problems.

10.3 The way science progresses

Most major scientific breakthroughs come unexpectedly. Sometimes, they can be the result of long, and targeted research reaching the goal, but in recent years, the big changes came from apparently lucky discoveries in combination with the ability to recognize and exploit said discovery. Hard work and determination is crucial, not only in making big milestone discoveries, but in advancing knowledge even the tiniest step. When we describe our research, we usually create a narrative that is logical and follows a red thread. When we do research, there usually is neither. Gut instinct and trial and error over and over are the primary methods of research. Progress is rarely linear, and failures commonly outnumber successes ten to one. And many times, it is not the carefully crafted experimental set-up, but a random observation that can determine the course of a project. Science is unpredictably and often frustrating. Science takes time and patience. And if anything, an open mind is the key ingredient for progress.

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Prilly/Zürich, July 2015

12 Curriculum vitae

Personal Information

Family name:	Morf
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Education

1999 – 2005	High school Zug, focus music, and chosen focus maths. High school exam on phytoestrogens in chemistry
2006 – 2009	University of Zurich, Bachelor of Science in biology, Minor in informatics
2009 – 2010	University of Zurich, Master of Science in Life Sciences (Fast Track), on the topic of "madd-2 regulates anchor cell invasion" with Prof. Dr. Alex Hajnal
2009 – 2015	University of Zurich, PhD thesis "The *Caenorhabditis elegans* homolog of the Opitz syndrome gene, *madd-2/ Mid1*, regulates anchor cell invasion during vulval development" and "Anchor Cell polarity negatively regulates vulval induction"

Oral presentation

C. elegans Development, Cell biology, & Gene Expression Meeting 2012, Madison, Wisconsin, USA: "MADD-2 Negatively Regulates Anchor Cell Invasion"

Poster presentations

1. EMBO Conference Series – C. elegans: Development and Gene Expression, 2010, EMBL advanced training center Heidelberg, Germany: "madd-2 regulates anchor cell invasion"

2. 18$^{\text{th}}$ International *C. elegans* meeting, 2011, University of California, Los Angeles, California, USA: "*madd-2* regulates anchor cell invasion"

Publications

1. "The dynactin complex enhances the speed of microtubule-dependent motions of adenovirus both towards and away from the nucleus" Engelke MF, Burckhardt CJ, Morf MK, Greber UF, Viruses. 2011 Mar;3(3):233-53. doi: 10.3390/v3030233.

2. "Coordinated lumen contraction and expansion during vulval tube morphogenesis in *Caenorhabditis elegans*" Farooqui S, Pellegrino MW, Rimann I, Morf MK, Müller L, Fröhli E, Hajnal A, Dev Cell. 2012 Sep 11;23(3):494-506. doi: 10.1016/j.devcel.2012.06.019.

3. "The *Caenorhabditis elegans* homolog of the Opitz syndrome gene, *madd-2/Mid1*, regulates anchor cell invasion during vulval development"
   Morf MK, Rimann I, Alexander M, Roy P, Hajnal A, Dev Biol. 2013 Feb 1;374(1):108-14. doi: 10.1016/j.ydbio.2012.11.019.

Teaching

1. 2011 – 2012: Supervision of the M.Sc. project of Louisa Müller, "LIN-3 EGF regulates dorsal lumen formation in the *C. elegans* vulva" at the Institute of Molecular Life Sciences, University of Zurich

2. 2009 – 2015: Supervision of various high and low level student courses

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